

US011976019B2

(12) **United States Patent**
Gatenyo et al.

(10) **Patent No.:** **US 11,976,019 B2**
(45) **Date of Patent:** **May 7, 2024**

(54) **CATIONIC LIPIDS FOR USE IN LIPID NANOPARTICLES**

(71) Applicant: **Acuitas Therapeutics, Inc.**, Vancouver (CA)

(72) Inventors: **Julia Gatenyo**, Vancouver (CA);
Xinyao Du, Richmond (CA)

(73) Assignee: **Acuitas Therapeutics, Inc.**, Vancouver (CA)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **17/378,083**

(22) Filed: **Jul. 16, 2021**

(65) **Prior Publication Data**

US 2022/0106257 A1 Apr. 7, 2022

Related U.S. Application Data

(60) Provisional application No. 63/188,996, filed on May 14, 2021, provisional application No. 63/052,815, filed on Jul. 16, 2020.

(51) **Int. Cl.**

C07C 219/06 (2006.01)

A61K 9/51 (2006.01)

A61K 39/12 (2006.01)

A61K 39/00 (2006.01)

(52) **U.S. Cl.**

CPC **C07C 219/06** (2013.01); **A61K 9/5123** (2013.01); **A61K 39/12** (2013.01); **A61K 2039/55555** (2013.01)

(58) **Field of Classification Search**

CPC **C07C 219/06**; **A61K 39/12**; **A61K 2039/55555**

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

2,856,420 A	10/1958	Crawford, Jr.
3,340,299 A	9/1967	Weintraub et al.
3,729,564 A	4/1973	Chang et al.
3,931,430 A	1/1976	Tada et al.
4,121,898 A	10/1978	Kirschnek et al.
4,491,583 A	1/1985	Cronin et al.
4,639,468 A	1/1987	Roncucci et al.
4,897,355 A	1/1990	Eppstein et al.
4,946,787 A	8/1990	Eppstein et al.
5,049,386 A	9/1991	Eppstein et al.
5,176,996 A	1/1993	Hogan et al.
5,420,032 A	5/1995	Marshall et al.
5,422,251 A	6/1995	Fresco
5,705,385 A	1/1998	Bally et al.
5,756,785 A	5/1998	O'Lenick, Jr.
5,789,538 A	8/1998	Rebar et al.
5,919,743 A	7/1999	O'Lenick, Jr.
5,925,523 A	7/1999	Dove et al.
5,965,542 A	10/1999	Wasan et al.
5,976,567 A	11/1999	Wheeler et al.
5,981,501 A	11/1999	Wheeler et al.

6,007,988 A	12/1999	Choo et al.
6,008,336 A	12/1999	Hanson et al.
6,013,453 A	1/2000	Choo et al.
6,013,813 A	1/2000	O'Lenick, Jr.
6,034,137 A	3/2000	Belloni et al.
6,077,509 A	6/2000	Weiner et al.

(Continued)

FOREIGN PATENT DOCUMENTS

CN	104876831 A	9/2015
EP	1 083 232 B1	2/2005

(Continued)

OTHER PUBLICATIONS

"ACS" "Can Cancer Be Prevented?" (www.cancer.org) Feb. 24, 2009, p. 1. (Year: 2009).*

Zhang, S. et al. "Fighting against Skin Aging: The Way from Bench to Bedside" Cell Transplantation 2018, vol. 27(5) 729-738 (Year: 2018).*

Akinc et al., "A combinatorial library of lipid-like materials for delivery of RNAi therapeutics," Nature Biotechnology 26(5):561-569, May 2008.

Akinc et al., "Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms," Mol. Ther. 18(7):1357-1364, 2010.

Alabi et al., "Multiparametric approach for the evaluation of lipid nanoparticles for siRNA delivery," PNAS 110(32):12881-12886, Aug. 6, 2013.

Alexidis et al., "Novel 1,4 Substituted Piperidine Derivatives. Synthesis and Correlation of Antioxidant Activity with Structure and Lipophilicity," J. Pharm. Pharmacol. 47:131-137, 1995.

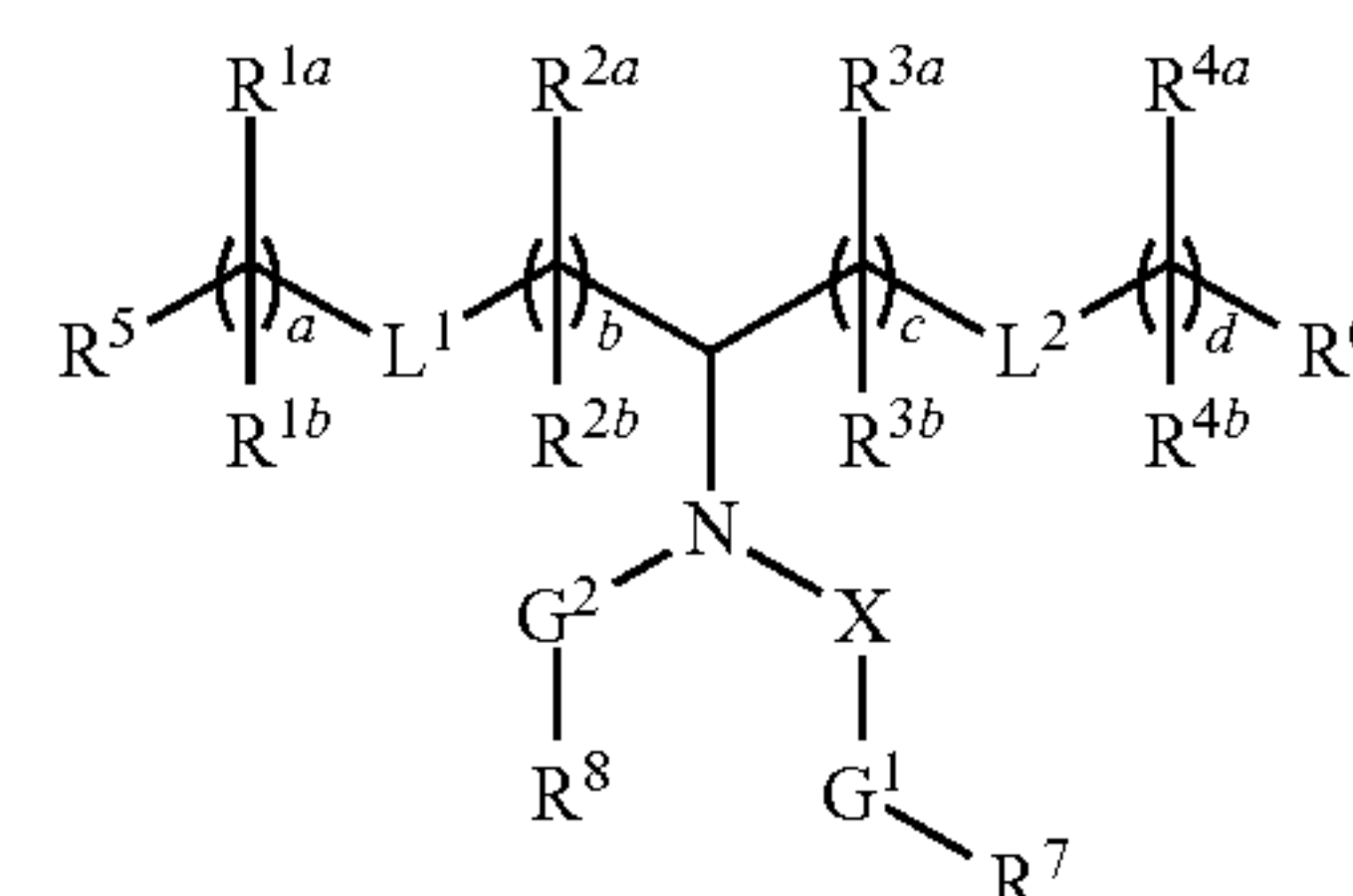
(Continued)

Primary Examiner — Andrew S Rosenthal

(74) *Attorney, Agent, or Firm* — Seed IP Law Group LLP

(57) **ABSTRACT**

Compounds are provided having the following structure:



(I)

or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein a, b, c, d, G¹, G², L¹, L², R^{1a}, R^{1b}, R^{2a}, R^{2b}, R^{3a}, R^{3b}, R^{4a}, R^{4b}, R⁵, R⁶, R⁷, R⁸ and X are as defined herein. Use of the compounds as a component of lipid nanoparticle formulations for delivery of a therapeutic agent, nanoparticles comprising the compounds and methods for their use and preparation are also provided.

27 Claims, No Drawings

(56)

References Cited

U.S. PATENT DOCUMENTS

6,107,286 A	8/2000	Byk et al.	8,563,314 B2	10/2013	Gregory et al.
6,140,081 A	10/2000	Barbas	8,569,256 B2	10/2013	Heyes et al.
6,140,466 A	10/2000	Barbas, III et al.	8,575,123 B2	11/2013	Manoharan et al.
6,200,759 B1	3/2001	Dove et al.	8,586,526 B2	11/2013	Gregory et al.
6,242,568 B1	6/2001	Barbas, III et al.	8,597,912 B2	12/2013	Collingwood et al.
6,300,321 B1	10/2001	Scherman et al.	8,623,618 B2	1/2014	Doyon et al.
6,333,433 B1	12/2001	Banerjee et al.	8,642,076 B2	2/2014	Manoharan et al.
6,410,248 B1	6/2002	Greisman et al.	8,691,750 B2	4/2014	Constien et al.
6,410,328 B1	6/2002	Maclachlan et al.	8,697,359 B1	4/2014	Zhang
6,453,242 B1	9/2002	Eisenberg et al.	8,703,489 B2	4/2014	Wang
6,458,381 B1	10/2002	Sourovoy et al.	8,722,082 B2	5/2014	Manoharan et al.
6,479,626 B1	11/2002	Kim et al.	8,748,089 B2	6/2014	Kariko et al.
6,503,717 B2	1/2003	Case et al.	8,754,062 B2	6/2014	De Fougerolles et al.
6,534,261 B1	3/2003	Cox, III et al.	8,771,985 B2	7/2014	Cui et al.
6,534,484 B1	3/2003	Wheeler et al.	8,772,453 B2	7/2014	Paschon et al.
6,586,410 B1	7/2003	Wheeler et al.	8,802,644 B2	8/2014	Chen et al.
6,599,692 B1	7/2003	Case et al.	8,822,668 B2	9/2014	Yaworski et al.
6,607,882 B1	8/2003	Cox, III et al.	8,835,108 B2	9/2014	Kariko et al.
6,620,794 B1	9/2003	O'Lenick, Jr. et al.	8,945,868 B2	2/2015	Collingwood et al.
6,689,558 B2	2/2004	Case	8,956,828 B2	2/2015	Bonini et al.
6,723,551 B2	4/2004	Kotin et al.	8,962,281 B2	2/2015	Doyon et al.
6,794,136 B1	9/2004	Eisenberg et al.	9,005,973 B2	4/2015	Cost et al.
6,815,432 B2	11/2004	Wheeler et al.	9,012,498 B2	4/2015	Manoharan et al.
6,824,978 B1	11/2004	Cox, III et al.	9,045,763 B2	6/2015	DeKolver et al.
6,833,252 B1	12/2004	Dujon et al.	9,139,554 B2	9/2015	Hope et al.
6,858,224 B2	2/2005	Wheeler et al.	9,150,847 B2	10/2015	Rebar
6,903,185 B2	6/2005	Kim et al.	9,200,266 B2	12/2015	Wang
6,933,113 B2	8/2005	Case et al.	9,234,016 B2	1/2016	Gregory et al.
6,979,539 B2	12/2005	Cox, III et al.	9,255,250 B2	2/2016	Gregory et al.
6,986,902 B1	1/2006	Chen et al.	9,352,042 B2	5/2016	Heyes et al.
6,989,264 B2	1/2006	Atkinson et al.	9,394,545 B2	7/2016	Miller
7,013,219 B2	3/2006	Case et al.	9,458,205 B2	10/2016	Gregory et al.
7,030,215 B2	4/2006	Liu et al.	9,604,908 B2	3/2017	Stanton et al.
7,067,317 B2	6/2006	Rebar et al.	9,682,922 B2	6/2017	Manoharan et al.
7,070,934 B2	7/2006	Cox, III et al.	9,737,619 B2	8/2017	Ansell et al.
7,074,596 B2	7/2006	Darzynkiewicz et al.	9,738,593 B2	8/2017	Ansell et al.
7,112,337 B2	9/2006	Huang et al.	9,750,824 B2	9/2017	Kariko et al.
7,153,949 B2	12/2006	Kim et al.	9,795,566 B2	10/2017	Oya et al.
7,163,824 B2	1/2007	Cox, III et al.	10,106,490 B2	10/2018	Du
7,217,509 B2	5/2007	Wolffe et al.	10,144,725 B2	12/2018	Brown
7,253,273 B2	8/2007	Collingwood	10,166,298 B2	1/2019	Ansell et al.
7,262,054 B2	8/2007	Jamieson et al.	10,221,127 B2	3/2019	Du et al.
7,271,002 B2	9/2007	Kotin et al.	10,723,692 B2	7/2020	Ansell et al.
7,361,635 B2	4/2008	Miller et al.	11,168,051 B2	11/2021	Du et al.
7,404,969 B2	7/2008	Chen et al.	2002/0062044 A1	5/2002	Banerjee et al.
7,419,817 B2	9/2008	Chiorini et al.	2003/0031704 A1	2/2003	Huang et al.
7,470,781 B2	12/2008	Crouzet et al.	2003/0153081 A1	8/2003	Tagawa et al.
7,745,651 B2	6/2010	Heyes et al.	2003/0232410 A1	12/2003	Liljedahl et al.
7,785,792 B2	8/2010	Wolffe et al.	2004/0002092 A1	1/2004	Arnould et al.
7,799,565 B2	9/2010	MacLachlan et al.	2004/0142025 A1	7/2004	MacLachlan et al.
7,803,397 B2	9/2010	Heyes et al.	2004/0142474 A1	7/2004	Mahato et al.
7,811,602 B2	10/2010	Cullis et al.	2005/0026157 A1	2/2005	Baltimore et al.
7,888,121 B2	2/2011	Urnov et al.	2005/0064474 A1	3/2005	Urnov et al.
7,893,302 B2	2/2011	Chen et al.	2005/0064595 A1	3/2005	MacLachlan et al.
7,901,708 B2	3/2011	MacLachlan et al.	2005/0208489 A1	9/2005	Carroll et al.
7,914,796 B2	3/2011	Miller et al.	2005/0222064 A1	10/2005	Vargeese et al.
7,923,542 B2	4/2011	Wolffe et al.	2005/0250723 A1	11/2005	Hoerr et al.
7,951,925 B2	5/2011	Ando et al.	2005/0267061 A1	12/2005	Martin
7,972,854 B2	7/2011	Miller et al.	2006/0063231 A1	3/2006	Li et al.
7,982,027 B2	7/2011	MacLachlan et al.	2006/0078552 A1	4/2006	Arnould et al.
8,034,376 B2	10/2011	Manoharan et al.	2006/0083780 A1	4/2006	Heyes et al.
8,034,598 B2	10/2011	Miller	2006/0100177 A1	5/2006	Nishimura et al.
8,071,370 B2	12/2011	Wolffe et al.	2006/0153836 A1	7/2006	Bailly et al.
8,110,379 B2	2/2012	DeKolver et al.	2006/0188490 A1	8/2006	Hoerr et al.
8,153,773 B2	4/2012	Jemielity et al.	2006/0188987 A1	8/2006	Guschin et al.
8,158,601 B2	4/2012	Chen et al.	2006/0206949 A1	9/2006	Arnould et al.
8,206,747 B2	6/2012	Zale et al.	2006/0240554 A1	10/2006	Chen et al.
8,278,036 B2	10/2012	Kariko et al.	2007/0042031 A1	2/2007	MacLachlan et al.
8,283,333 B2	10/2012	Yaworski et al.	2007/0117128 A1	5/2007	Smith et al.
8,293,276 B2	10/2012	Troiano et al.	2007/0218528 A1	9/2007	Miller
8,318,208 B1	11/2012	Zale et al.	2008/0020058 A1	1/2008	Chen et al.
8,318,211 B2	11/2012	Zale et al.	2008/0025944 A1	1/2008	Hoerr et al.
8,329,070 B2	12/2012	MacLachlan et al.	2008/0131962 A1	6/2008	Miller
8,409,861 B2	4/2013	Guschin et al.	2008/0159996 A1	7/2008	Ando et al.
8,466,122 B2	6/2013	Heyes et al.	2009/0068164 A1	3/2009	Segal et al.
			2009/0086558 A1	4/2009	Do
			2009/0136465 A1	5/2009	Merenick et al.
			2009/0209037 A1	8/2009	Tagawa et al.
			2009/0247608 A1	10/2009	Manoharan et al.

(56)

References Cited

U.S. PATENT DOCUMENTS

2009/0263407	A1	10/2009	Dande et al.	2013/0259879	A1	10/2013	Baumhof et al.
2009/0285881	A1	11/2009	Dande et al.	2013/0261172	A1	10/2013	Kariko et al.
2009/0305346	A1	12/2009	Miller	2013/0266640	A1	10/2013	de Fougerolles et al.
2009/0324584	A1	12/2009	Hoerr et al.	2013/0280283	A1	10/2013	Lorenz et al.
2010/0036115	A1	2/2010	Beigelman et al.	2013/0280305	A1	10/2013	Kuboyama et al.
2010/0068285	A1	3/2010	Zale et al.	2013/0295043	A1	11/2013	Kallen et al.
2010/0068286	A1	3/2010	Troiano et al.	2013/0323269	A1	12/2013	Manoharan et al.
2010/0069426	A1	3/2010	Zale et al.	2013/0336998	A1	12/2013	Kallen et al.
2010/0087337	A1	4/2010	Dewitt	2013/0338210	A1	12/2013	Manoharan et al.
2010/0104645	A1	4/2010	Ali et al.	2014/0017212	A1	1/2014	Rebar et al.
2010/0104655	A1	4/2010	Zale et al.	2014/0044772	A1	2/2014	MacLachlan et al.
2010/0189729	A1	7/2010	Hoerr et al.	2014/0120622	A1	5/2014	Gregory et al.
2010/0203076	A1	8/2010	Fotin-Mleczek et al.	2014/0121393	A1	5/2014	Manoharan et al.
2010/0218264	A1	8/2010	Cui et al.	2014/0134260	A1	5/2014	Heyes et al.
2010/0285112	A1	11/2010	Novobrantseva et al.	2014/0179761	A1	6/2014	Manoharan et al.
2010/0291156	A1	11/2010	Barner et al.	2014/0256785	A1	9/2014	Manoharan et al.
2010/0305196	A1	12/2010	Probst et al.	2014/0294937	A1	10/2014	MacLachlan et al.
2010/0324120	A1	12/2010	Chen et al.	2014/0295449	A1	10/2014	Ciufolini et al.
2011/0045473	A1	2/2011	De Fougerolles et al.	2014/0308304	A1	10/2014	Manoharan et al.
2011/0091525	A1	4/2011	Heyes et al.	2014/0323548	A1	10/2014	Budzik et al.
2011/0097720	A1	4/2011	Ciufolini et al.	2015/0037326	A1	2/2015	Butler-Ransohoff et al.
2011/0145940	A1	6/2011	Voytas et al.	2015/0050302	A1	2/2015	Thess
2011/0200582	A1	8/2011	Baryza et al.	2015/0056705	A1	2/2015	Conway et al.
2011/0201055	A1	8/2011	Doyon et al.	2015/0057340	A1	2/2015	Thess et al.
2011/0250225	A1	10/2011	Fotin-Mleczek et al.	2015/0093413	A1	4/2015	Thess et al.
2011/0256175	A1	10/2011	Hope et al.	2015/0118183	A1	4/2015	Baumhof
2011/0262491	A1	10/2011	Keegan et al.	2015/0118264	A1	4/2015	Baumhof et al.
2011/0262527	A1	10/2011	Heyes et al.	2015/0159172	A1	6/2015	Miller et al.
2011/0265198	A1	10/2011	Gregory et al.	2015/0165006	A1	6/2015	Thess et al.
2011/0274759	A1	11/2011	Troiano et al.	2015/0184195	A1	7/2015	Thess et al.
2011/0294717	A1	12/2011	Ali et al.	2015/0203446	A1	7/2015	Manoharan et al.
2011/0300205	A1	12/2011	Geall et al.	2015/0218554	A1	8/2015	Thess
2011/0301073	A1	12/2011	Gregory et al.	2015/0306249	A1	10/2015	Baumhof et al.
2011/0305770	A1	12/2011	Zhao et al.	2015/0335708	A1	11/2015	Kwak et al.
2011/0311582	A1	12/2011	Manoharan et al.	2015/0376115	A1	12/2015	Ansell et al.
2011/0311583	A1	12/2011	Manoharan et al.	2016/0009637	A1	1/2016	Manoharan et al.
2012/0004293	A1	1/2012	Zale et al.	2016/0024474	A1	1/2016	Cost et al.
2012/0017290	A1	1/2012	Cui et al.	2016/0095924	A1	4/2016	Hope et al.
2012/0021043	A1	1/2012	Kramps et al.	2016/0130345	A1	5/2016	Fotin-Mleczek et al.
2012/0027796	A1	2/2012	Manoharan et al.	2016/0166668	A1	6/2016	Kallen et al.
2012/0027803	A1	2/2012	Manoharan et al.	2016/0166678	A1	6/2016	Kallen et al.
2012/0040398	A1	2/2012	Miller	2016/0166710	A1	6/2016	Baumhof
2012/0046478	A1	2/2012	Manoharan et al.	2016/0166711	A1	6/2016	Schnee et al.
2012/0058144	A1	3/2012	Manoharan et al.	2016/0168207	A1	6/2016	Kramps et al.
2012/0058188	A1	3/2012	Manoharan et al.	2016/0235864	A1	8/2016	Schlake et al.
2012/0060230	A1	3/2012	Collingwood et al.	2016/0263278	A1*	9/2016	Grinstaff C08L 43/02
2012/0095075	A1	4/2012	Manoharan et al.	2016/0304883	A1	10/2016	Grund et al.
2012/0101148	A1	4/2012	Aking et al.	2016/0326548	A1	11/2016	Cost
2012/0128760	A1	5/2012	Manoharan et al.	2016/0361411	A1	12/2016	Gindy et al.
2012/0172411	A1	7/2012	Heyes et al.	2016/0376224	A1	12/2016	Du et al.
2012/0177724	A1	7/2012	Irvine et al.	2017/0027803	A1	2/2017	Agrawal et al.
2012/0178702	A1	7/2012	Huang et al.	2017/0029847	A1	2/2017	Thess
2012/0183602	A1	7/2012	Chen et al.	2017/0119904	A1	5/2017	Ansell et al.
2012/0195936	A1	8/2012	Rudolph et al.	2017/0157268	A1	6/2017	Ansell et al.
2012/0202871	A1	8/2012	Heyes et al.	2017/0173080	A1	6/2017	Lee et al.
2012/0207845	A1	8/2012	Sung et al.	2017/0211075	A1	7/2017	Lee et al.
2012/0225434	A1	9/2012	Ciufolini et al.	2017/0252430	A1	9/2017	Fotin-Mleczek et al.
2012/0244207	A1	9/2012	Fitzgerald et al.	2017/0266292	A1	9/2017	Luo et al.
2012/0251618	A1	10/2012	Schrum et al.	2017/0283367	A1	10/2017	Du
2012/0264810	A1	10/2012	Lin et al.	2017/0326225	A1	11/2017	Rauch et al.
2012/0276209	A1	11/2012	Cullis et al.	2018/0044687	A1	2/2018	Thess et al.
2012/0288541	A1	11/2012	Zale et al.	2018/0064827	A1	3/2018	Conway et al.
2012/0295832	A1	11/2012	Constien et al.	2018/0087072	A1	3/2018	Miller et al.
2013/0017223	A1	1/2013	Hope et al.	2018/0125952	A1	5/2018	Fotin-Mleczek et al.
2013/0022649	A1	1/2013	Yaworski et al.	2018/0126003	A1	5/2018	Hoerr
2013/0108685	A1	5/2013	Kuboyama et al.	2018/0148727	A1	5/2018	Grund et al.
2013/0122104	A1	5/2013	Yaworski et al.	2018/0185516	A1	7/2018	Ansell et al.
2013/0122591	A1	5/2013	Cost et al.	2018/0214537	A1	8/2018	Mutzke et al.
2013/0123338	A1	5/2013	Heyes et al.	2018/0237786	A1	8/2018	Schlake et al.
2013/0129754	A1	5/2013	Thess et al.	2018/0296663	A1	10/2018	Hipp et al.
2013/0129811	A1	5/2013	Kuboyama et al.	2018/0303925	A1	10/2018	Weissman et al.
2013/0137104	A1	5/2013	Cost et al.	2018/0312545	A1	11/2018	Baumhof et al.
2013/0177690	A1	7/2013	Regar	2019/0022247	A1	1/2019	Ansell et al.
2013/0177983	A1	7/2013	Rebar	2019/0024096	A1	1/2019	Schmid et al.
2013/0196373	A1	8/2013	Gregory et al.	2019/0160164	A1	5/2019	Rauch et al.
				2019/0270697	A1	9/2019	Ansell et al.
				2019/0274968	A1	9/2019	Weissman et al.
				2019/0314524	A1	10/2019	Ansell et al.
				2019/0358314	A1	11/2019	Weissman et al.

(56)

References Cited

U.S. PATENT DOCUMENTS

2019/0359556 A1 11/2019 Du et al.
 2020/0046838 A1 2/2020 Ansell et al.
 2020/0121809 A1 4/2020 Hope et al.
 2020/0163878 A1 5/2020 Baumhof et al.
 2020/0172472 A1 6/2020 Du
 2020/0283372 A1 9/2020 Du
 2021/0107861 A1 4/2021 Ansell et al.
 2021/0122702 A1 4/2021 Du
 2021/0122703 A1 4/2021 Du
 2021/0128488 A1 5/2021 Du
 2021/0251898 A1 8/2021 Baumhof et al.
 2021/0395188 A1 12/2021 Ansell

FOREIGN PATENT DOCUMENTS

GB 1 277 947 A 6/1972
 GB 2 338 237 A 12/1999
 JP H05-331118 A 12/1993
 JP H10-3643 A 1/1998
 JP 2001-338416 A 12/2001
 JP 4681425 B2 5/2011
 WO 87/07183 A1 12/1987
 WO 95/19431 A1 7/1995
 WO 96/06166 A1 2/1996
 WO 97/03939 A1 2/1997
 WO 98/16599 A1 4/1998
 WO 98/37186 A1 8/1998
 WO 98/53057 A1 11/1998
 WO 98/53058 A1 11/1998
 WO 98/53059 A1 11/1998
 WO 98/53060 A1 11/1998
 WO 98/54311 A1 12/1998
 WO 99/05094 A1 2/1999
 WO 99/33493 A1 7/1999
 WO 00/27878 A1 5/2000
 WO 00/30444 A1 6/2000
 WO 01/07548 A1 2/2001
 WO 01/48233 A1 7/2001
 WO 01/60970 A2 8/2001
 WO 01/88197 A2 11/2001
 WO 02/016536 A1 2/2002
 WO 02/099084 A2 12/2002
 WO 03/016496 A2 2/2003
 WO 03/053409 A1 7/2003
 WO 2005/060934 A1 7/2005
 WO 2006/138380 A2 12/2006
 WO 2007/014275 A2 2/2007
 WO 2007/024708 A2 3/2007
 WO 2008/103276 A2 8/2008
 WO 2008/121949 A1 10/2008
 WO 2009/086558 A1 7/2009
 WO 2009/127060 A1 10/2009
 WO 2009/132131 A1 10/2009
 WO 2010/005721 A2 1/2010
 WO 2010/005723 A2 1/2010
 WO 2010/005725 A2 1/2010
 WO 2010/005726 A2 1/2010
 WO 2010/005740 A2 1/2010
 WO 2010/021865 A1 2/2010
 WO 2010/030763 A2 3/2010
 WO 2010/042877 A1 4/2010
 WO 2010/048536 A2 4/2010
 WO 2010/054384 A1 5/2010
 WO 2010/054401 A1 5/2010
 WO 2010/054405 A1 5/2010
 WO 2010/054406 A1 5/2010
 WO 2010/057150 A1 5/2010
 WO 2010/062322 A2 6/2010
 WO 2010/079430 A1 7/2010
 WO 2010/080724 A1 7/2010
 WO 2010/088537 A2 8/2010
 WO 2010/129709 A1 11/2010
 WO 2011/022460 A1 2/2011
 WO 2011/043913 A2 4/2011
 WO 2011/075656 A1 6/2011

WO 2011/076807 A2 6/2011
 WO 2011/084513 A2 7/2011
 WO 2011/084521 A2 7/2011
 WO 2011/090965 A1 7/2011
 WO 2011/094198 A1 8/2011
 WO 2011/127255 A1 10/2011
 WO 2011/141703 A1 11/2011
 WO 2011/141705 A1 11/2011
 WO 2011/143230 A1 11/2011
 WO 2011/149733 A2 12/2011
 WO 2011/153120 A1 12/2011
 WO 2011/153493 A2 12/2011
 WO 2012/000104 A1 1/2012
 WO 2012/006378 A1 1/2012
 WO 2012/006380 A2 1/2012
 WO 2012/016184 A2 2/2012
 WO 2012/019630 A1 2/2012
 WO 2012/030901 A1 3/2012
 WO 2012/031043 A1 3/2012
 WO 2012/031046 A2 3/2012
 WO 2012/040184 A2 3/2012
 WO 2012/044638 A1 4/2012
 WO 2012/054365 A2 4/2012
 WO 2012/054923 A2 4/2012
 WO 2012/061259 A2 5/2012
 WO 2012/068176 A1 5/2012
 WO 2013/014073 A1 1/2013
 WO 2013/016058 A1 1/2013
 WO 2013/044008 A2 3/2013
 WO 2013/059496 A1 4/2013
 WO 2013/086322 A1 6/2013
 WO 2013/086354 A1 6/2013
 WO 2013/086373 A1 6/2013
 WO 2013/143555 A1 10/2013
 WO 2014/008334 A1 1/2014
 WO 2014/028487 A1 2/2014
 WO 2014/089239 A1 6/2014
 WO 2014/152200 A1 9/2014
 WO 2014/153163 A1 9/2014
 WO 2014/160243 A1 10/2014
 WO 2014/160284 A1 10/2014
 WO 2015/074085 A1 5/2015
 WO 2015/123576 A2 8/2015
 WO 2015/130584 A2 9/2015
 WO 2015/164674 A1 10/2015
 WO 2015/177752 A1 11/2015
 WO 2016/010840 A1 1/2016
 WO 2016/014794 A1 1/2016
 WO 2016/183298 A1 11/2016
 WO WO-2016176330 A1 * 11/2016 A61K 31/7115
 WO 2017/004143 A1 1/2017
 WO 2017/021546 A1 2/2017
 WO 2017/048770 A1 3/2017
 WO 2017/049245 A2 3/2017
 WO 2017/074526 A1 5/2017
 WO 2017/075531 A1 5/2017
 WO 2017/112865 A1 6/2017
 WO 2017/117528 A1 7/2017
 WO 2017/140905 A1 8/2017
 WO 2017/173054 A1 10/2017
 WO 2017/182634 A1 10/2017
 WO 2017/194454 A1 11/2017
 WO 2017/201332 A1 11/2017
 WO 2016/176330 A1 3/2018
 WO 2018/078053 A1 5/2018
 WO 2018/081638 A1 5/2018
 WO 2018/191657 A1 10/2018
 WO 2018/191719 A1 10/2018
 WO 2018/200943 A1 11/2018
 WO 2019/089828 A1 5/2019
 WO 2021/030701 A1 2/2021
 WO 2022/016070 A1 1/2022

OTHER PUBLICATIONS

Anderson et al., "Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation," *Nucleic Acids Res.* 38(17):5884-5892, 2010.

(56)

References Cited

OTHER PUBLICATIONS

- Anderson et al., "Nucleoside modifications in RNA limit activation of 2'-5'-oligoadenylate synthetase and increase resistance to cleavage by RNase L.," *Nucleic Acids Research* 39(21):9329-9338, 2011.
- Ansell et al., "Application of Oligo-(14-amino-3,6,9,12-tetraoxatetradecanoic acid) Lipid Conjugates as Steric Barrier Molecules in Liposomal Formulations," *Bioconjugate Chem.* 10:653-666, 1999.
- Ansell et al., "Novel Lipids and Lipid Nanoparticle Formulations for Delivery of Nucleic Acids," U.S. Appl. No. 15/337,434, filed Oct. 28, 2016, 87 pages.
- Argast et al., "I-PPOL and I-CREL Homing Site Sequence Degeneracy Determined by Random Mutagenesis and Sequential in vitro Enrichment," *J. Mol. Biol.* 280:345-353, 1998.
- Ashworth et al., "Computational Redesign of Endonuclease DNA Binding and Cleavage Specificity," *Nature* 441:656-659, 2006.
- Basha et al., "Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells," *Mol. Ther.* 19(12):2186-2200, 2011.
- Beerli et al., "Engineering Polydactyl Zinc-Finger Transcription Factors," *Nature Biotechnology* 20:135-141, 2002.
- Belfort et al., "Homing Endonucleases: Keeping the House in Order," *Nucleic Acids Research* 25:3379-3388, 1997.
- Belliveau et al., "Microfluidic Synthesis of Highly Potent Limit-size Lipid Nanoparticles for In Vivo Delivery of siRNA," *Mol. Ther. Nucleic Acids* 1:e37, 2012 (9 pages).
- Beurdeley et al., "Compact Designer TALENs for Efficient Genome Engineering," *Nat. Commun.* 1-8: DOI: 10.1038/ncomms2782, 2013.
- Bhattacharya et al., "Synthesis, Thermotropic Behavior, and Permeability Properties of Vesicular Membranes Composed of Cationic Mixed-Chain Surfactants," *Langmuir* 11:4748-4757, 1995.
- Boch et al., "Breaking the Code of DNA Binding Specificity of TAL-Type III Effectors," *Science* 326:1509-1512, 2009.
- Boissel et al., "MegaTALs: A Rare-Cleaving Nuclease Architecture for Therapeutic Genome Engineering," *Nucl Acid Res* 42(4):2591-2601, 2014.
- Bonas et al., "Genetic and Structural Characterization of the Avirulence Gene avrBS3 from *Xanthomonas campestris* PV. *vesicatoria*," *Mol Gen Genet* 218:127-136, 1989.
- Brito et al., "A Cationic Nanoemulsion for the Delivery of Next-generation RNA Vaccines," *Molecular Therapy* 22(12):2118-2129, 2014.
- Cattanach et al., "Studies in the Indole Series. Part IV. Tetrahydro-1H-pyrido[4,3-b]-indoles as Serotonin Antagonists," *J. Chem Soc. (C)*:1235-1243, 1968.
- Chen et al., "Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA," *Journal of Controlled Release* 235:236-244, 2016.
- Chen et al., "Rapid Discovery of Potent siRNA-Containing Lipid Nanoparticles Enabled by Controlled Microfluidic Formulation," *J. Am. Chem. Soc.* 134:6948-6951, 2012.
- Chevalier et al., "Design, Activity, and Structure of a Highly Specific Artificial Endonuclease," *Molecular Cell* 10:895-905, 2002.
- Choo et al., "Advances in Zinc Finger Engineering," *Current Opinion in Structural Biology* 10:411-416, 2000.
- Cook et al., "Synthesis and Characterization of cis-Dioxomolybdenum(VI) Complexes with Sterically Bulky Tripodal Tetradentate Ligands," *Inorganica Chimica Acta* 144:81-87, 1988.
- Cullis et al., "Lipid Nanoparticle Systems for Enabling Gene Therapies," *Molecular Therapy* 25(7):1467-1475, 2017.
- Dujon et al., "Mobile introns: definition of terms and recommend nomenclature," *Gene* 82:115-118, 1989.
- Durbin et al., "RNAs Containing Modified Nucleotides Fail to Trigger RIG-I Conformational Changes for Innate Immune Signaling," *mBio* 7(5):e00833-16, 2016 (11 pages).
- Epinat et al., "A novel engineered meganuclease induces homologous recombination in yeast and mammalian cells," *Nucleic Acids Research* 31:2952-2962, 2003.
- Ernsting et al., "Factors controlling the pharmacokinetics, biodistribution and intratumoral penetration of nanoparticles," *Journal of Controlled Release* 172:782-794, 2013.
- Fagerlund et al., "The Cpf1 CRISPR-Cas Protein Expands Genome-Editing Tools," *Genom Bio* 16:251, 2015 (3 pages).
- Falcone et al., "Both the 5' Untranslated Region and the Sequences Surrounding the Start Site Contribute to Efficient Initiation of Translation In Vitro," *Molecular and Cellular Biology* 11(5):2656-2664, 1991.
- Frisch et al., "A New Triantennary Galactose-Targeted PEGylated Gene Carrier, Characterization of Its Complex with DNA, and Transfection of Hepatoma Cells," *Bioconjugate Chem.* 15: 754-764, 2004.
- Gimble et al., "Substrate Recognition and Induced DNA Distortion by the P1-SceI Endonuclease, an Enzyme Generated By Protein Splicing," *J. Mol. Biol.* 263:163-180, 1996.
- Gronquist et al., "Synthesis of Unsaturated Polyazamacrolides from the Ladybird Beetle *Subcoccinella vigintiquatuorpuntata*," *J. Org. Chem.* 66:1075-1081, 2001.
- Guilinger et al., "Fusion of Catalytically Inactive Cas9 to FokI Nuclease Improves the Specificity of Genome Modification," *Nature Biotech* 32(6):577-582, 2014.
- Guo et al., "Directed Evolution of an Enhanced and Highly Efficient FokI Cleavage Domain for Zinc Finger Nucleases," *J. Mol. Biol.* 400(1):96-107, 2010.
- Haft et al., "A Guild of 45 CRISPR-Associated (Cas) Protein Families and Multiple CRISPR/Cas Subtypes Exist in Prokaryotic Genomes," *PLoS Computational Biology* 1(6):e60, 2005.
- Han et al., "Synthesis and Properties of Di-Chain Esterquat Surfactants," *J. Surfact Deterg.* 18: 91-95, 2015.
- Hancock et al., "Monoalkylaminopropanols and Butanols and their Esters," *J. Am. Chem. Soc.* 66(10):1738-1747, 1944.
- Hassett et al., "Optimization of Lipid Nanoparticles for Intramuscular Administration of mRNA Vaccines," *Molecular Therapy: Nucleic Acids* 15:1-11, 2019.
- Hekele et al., "Rapidly produced SAM® vaccine against H7N9 influenza is immunogenic in mice," *Emerging Microbes and Infections* 2:e52, 2013 (7 pages).
- Heuer et al., "Repeat Domain Diversity of avrBs3-Like Genes in *Ralstonia solanacearum* Strains and Association with Host Preferences in the Field," *Applied and Environmental Microbiology* 73(13):4379-4384, 2007.
- International Preliminary Report on Patentability, dated Apr. 30, 2019, for International Application No. PCT/EP2017/077517, 13 pages.
- International Search Report and Written Opinion, dated Apr. 16, 2018, for International Application No. PCT/EP2017/077517, 21 pages.
- Isalan et al., "A rapid, generally applicable method to engineer zinc fingers illustrated by targeting the HIV-1 promoter," *Nat. Biotechnol* 19(7):656-660, 2001.
- Jansen et al., "Identification of Genes that are Associated with DNA Repeats in Prokaryotes," *Molecular Microbiology* 43(6):1565-1575, 2002.
- Jasin, "Genetic Manipulation of Genomes with Rare-Cutting Endonucleases," *Trends Genet* 12:224-228, 1996.
- Jayaraman et al., "Maximizing the Potency of siRNA Lipid Nanoparticles for Hepatic Gene Silencing In Vivo," *Angew. Chem. Int. Ed.* 51(34):8529-8533, XP055063645, 2012.
- John et al., "Multi-antigenic human cytomegalovirus mRNA vaccines that elicit potent humoral and cell-mediated immunity," *Vaccine* 36:1689-1699, 2018.
- Karikó et al., "Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA," *Nucleic Acids Research* 39(21):e142, 2011.
- Karikó et al., "Incorporation of Pseudouridine into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability," *Mol. Ther.* 16(11):1833-1840, 2008.
- Karikó et al., "Increased Erythropoiesis in Mice Injected with Submicrogram Quantities of Pseudouridine-containing mRNA Encoding Erythropoietin," *Mol. Ther.* 20(5):948-953, 2012.

(56)

References Cited

OTHER PUBLICATIONS

- Karikó et al., "Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA," *Immunity* 23:165-175, 2005.
- Kay et al., "A Bacterial Effector Acts as a Plant Transcription Factor and Induces a Cell Size Regulator," *Science* 318:648-651, 2007.
- Kim et al., "Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain," *PNAS USA* 93(3):1156-1160, 1996.
- Kim et al., "Highly Efficient RNA-Guided Genome Editing in Human Cells via Delivery of Purified Cas9 Ribonucleoproteins," *Genome Research* 24(6):1012-1019, 2014.
- Kormann et al., "Expression of therapeutic proteins after delivery of chemically modified mRNA in mice," *Nature Biotechnology* 29(2):154-157, 2011.
- Lee et al., "Lipid nanoparticle siRNA systems for silencing the androgen receptor in human prostate cancer in vivo," *Int. J. Cancer* 131(5):E781-E790, 2012.
- Leroueil et al., "Wide Varieties of Cationic Nanoparticles Induce Defects in Supported Lipid Bilayers," *Nano Letters* 8(2):420-424, 2008.
- Leung et al., "Lipid Nanoparticles Containing siRNA Synthesized by Microfluidic Mixing Exhibit an Electron-Dense Nanostructured Core," *J. Phys. Chem. C. Nanomater. Interfaces* 116(34):18440-18450, 2012.
- Leung et al., "Microfluidic Mixing: A General Method for Encapsulating Macromolecules in Lipid Nanoparticle Systems," *J. Phys. Chem. B* 119:8698-8706, 2015.
- Li et al., "ATRP in Waterborne Miniemulsion via a Simultaneous Reverse and Normal Initiation Process," *Macromolecules* 37(6):2106-2112, 2004.
- Mahon et al., "A combinatorial approach to determine functional group effects on lipidoid-mediated siRNA delivery," *Bioconjug Chem.* 21(8):1448-1454, 2010. (17 pages).
- Maier et al., "Biodegradable lipids enabling rapidly eliminated lipid nanoparticles for systemic delivery of RNAi therapeutics," *Mol. Ther.* 21(8):1570-1578, 2013.
- Makarova et al., "A DNA Repair System Specific for Thermophilic Archaea and Bacteria Predicted by Genomic Context Analysis," *Nucleic Acids Research* 30(2):482-496, 2002.
- Makarova et al., "A Putative RNA-Interference-Based Immune System in Prokaryotes: Computational Analysis of the Predicted Enzymatic Machinery, Functional Analogies with Eukaryotic RNAi, and Hypothetical Mechanisms of Action," *Biology Direct* 1:7, 2006.
- Marchi-Artzner et al., "Adhesion of Arg-Gly-Asp (RGD) Peptide Vesicles onto an Integrin Surface: Visualization of the Segregation of RGD Ligands into the Adhesion Plaques by Fluorescence," *Langmuir* 19:835-841, 2003.
- Masuda et al., "Envelope-type lipid nanoparticles incorporating a short PEG-lipid conjugate for improved control of intracellular trafficking and transgene transcription," *Biomaterials* 30:4806-4814, 2009.
- McCaffrey et al., "CRISPR-CAS9 D10A Nickase Target-Specific Fluorescent Labeling of Double Stand DNA for Whole Genome Mapping and Structural Variation Analysis," *Nucleic Acids Res* 44(2):e11.doi: 10.1093/ar/gkv878, 2015.
- Miller et al., "An improved zinc-finger nuclease architecture for highly specific genome editing," *Nat. Biotechnology* 25:778-785, 2007.
- Moscou et al., "A Simple Cipher Governs Data Recognition by TAL Effectors," *Science* 326:1501, 2009 (2 pages).
- Mui et al., "Influence of Polyethylene Glycol Lipid Desorption Rates on Pharmacokinetics and Pharmacodynamics of siRNA Lipid Nanoparticles," *Mol. Ther. Nucleic Acids* 2:e139, 2013 (8 pages).
- Nguyen et al., "Lipid-derived nanoparticles for immunostimulatory RNA adjuvant delivery", Proceedings of the National Academy of Sciences 109(14):E797-E803, 2012.
- Nishida, "Disk-shaped magnetic recording medium," Caplus Database, Accession No. 2001:881906, 2001 (1 page).
- Olovnikov et al., "Bacterial Argonaute samples the transcriptome to identify foreign DNA," *Mol. Cell.* 51(5):594-605, 2013.
- Pabo et al., "Design and Selection of Novel Cys₂-His₂ Zinc Finger Proteins," *Ann. Rev. Biochem.* 70:313-340, 2001.
- Pâques et al., "Meganucleases and DNA Double-Strand Break-Induced Recombination: Perspectives for Gene Therapy," *Current Gene Therapy* 7:49-66, 2007.
- Pardi et al., "Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes," *Journal of Controlled Release* 217:345-351, 2015.
- Petsch et al., "Protective efficacy of in vitro synthesized, specific mRNA vaccines against influenza A virus infection," *Nature Biotechnology* 30(12):1210-1216, 2012 (9 pages).
- Perez et al., "Establishment of HIV-1 Resistance in CD4+ T Cells by Genome Editing Using Zinc-Finger Nucleases," *Nature Biotechnology* 26(7):808-816, 2008.
- Perler et al., "Protein splicing elements: inteins and exteins a definition of terms and recommended nomenclature," *Nucleic Acids Research* 22:1125-1127, 1994.
- Poveda et al., "Establishing Preferred Product Characterization for the Evaluation of RNA Vaccine Antigens," *Vaccines* 7:131, 2019 (14 pages).
- Rajesh et al., "Dramatic Influence of the Orientation of Linker between Hydrophilic and Hydrophobic Lipid Moiety in Liposomal Gene Delivery," *Journal of the American Chemical Society* 129(37):11408-11420, 2007.
- Ran et al., "In Vivo Genome Editing Using *Staphylococcus aureus* Cas9," *Nature* 520:186-194, 2015.
- Russell et al., "The Stability of Human β -Globin mRNA is Dependent on Structural Determinants Positioned Within Its 3' Untranslated Region," *Blood* 87:5314-5323, 1996.
- Schar et al., "Long Chain Linear Fatty Alcohols from Ziegler-Synthesis, their Mixtures, Derivatives and Use," IP.com Prior Art Database Technical Disclosure, Jan. 17, 2011, 39 pages.
- Schnee et al., "An mRNA Vaccine Encoding Rabies Virus Glycoprotein Induces Protection against Lethal Infection in Mice and Correlates of Protection in Adult and Newborn Pigs," *PLoS Negl. Trop. Dis.* 10(6):e0004746, 2016 (20 pages).
- Schornack et al., "Gene-for-Gene-Mediated Recognition of Nuclear-Targeted AvrBs3-Like Bacterial Effector Proteins," *Journal of Plant Physiology* 163(3):256-272, 2006.
- Segal et al., "Custom DNA-binding proteins come of age: polydactyl zinc-finger proteins," *Current Opinion Biotechnology* 12:632-637, 2001.
- Semple et al., "Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo," *Advanced Drug Delivery Reviews* 32:3-17, 1998.
- Semple et al., "Rational design of cationic lipids for siRNA delivery," *Nature Biotechnology* 28(2):172-176, 2010. (26 pages).
- Sheng et al., "Structure-Based cleavage mechanism of thermophilus argonaute dna guide strand-mediated dna target cleavage," *Proc. Natl. Acad. Sci. USA* 111(2):652-657, 2014.
- Smietanski et al., "Structural analysis of human 2'-O-ribose methyltransferases involved in mRNA cap structure formation," *Nature Communications* 5:3004, 2014.
- Swarts et al., "DNA-Guided DNA Interference by a Prokaryotic Argonaute," *Nature* 507(7491):258-261, 2014.
- Szebeni et al., "Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: Prediction and prevention," *Advanced Drug Delivery Reviews* 63:1020-1030, 2011.
- Szebeni et al., "Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs," *Biochemical and Biophysical Research Communications* 468:490-497, 2015.
- Szebeni, "Complement activation-related pseudoallergy: A stress reaction in blood triggered by nanomedicines and biologicals," *Molecular Immunology* 61:163-173, 2014.
- Szolcsányi et al., "Short racemic syntheses of calvine and epicalvine," *Tetrahedron Letters* 49:1357-1360, 2008.
- Tam et al., "Advances in Lipid Nanoparticles for siRNA Delivery," *Pharmaceutics* 5:498-507, 2013.
- Tam et al., "Small molecule ligands for enhanced intracellular delivery of lipid nanoparticle formulations of siRNA," *Nanomedicine* 9(5):665-674, 2013.

(56)

References Cited

OTHER PUBLICATIONS

Tekmira Pharmaceuticals Corp, Form 20-F, Edgar Online, filed Mar. 27, 2013, 298 pages.

Tekmira, "Tekmira and Alnylam Restructure Relationship and Settle All Litigation," Tekmira Pharmaceuticals Corporation, Nov. 12, 2012, 3 pages.

Torrecilla et al., "Lipid Nanoparticles as Carriers for RNAi against Viral Infections: Current Status and Future Perspectives," BioMed Research International 2014:Article ID 161794, 17 pages.

Urnov et al., "Highly Efficient Endogenous Human Gene Correction Using Designed Zinc-Finger Nucleases," *Nature* 435(7042):646-651, 2005.

Vanderah et al., "Oligo(ethylene oxide) Self-Assembled Monolayers with Self-Limiting Packing Densities for the Inhibition of Nonspecific Protein Adsorption," *Langmuir* 25(9):5026-5030, 2009.

Van Doren et al., "Structure-Property Relationships in D-Glucitol Derivatives with Two Geminal Hydrocarbon Chains," *J. Mater. Chem.* 5(12):2153-2160, 1995.

Vogel et al., "A Bacterial Seek-and-Destroy System for Foreign DNA," *Science* 344(6187):972-73, 2014.

Wah et al., "Structure of the Multimodular Endonuclease FokI Bound to DNA," *Nature* 388:97-100, 1997.

Wang et al., "Lipid Nanoparticles for Ocular Gene Delivery," *J. Funct. Biomat* 6(2):379-394, 2015.

Wang et al., "Composite Nanoparticles for Gene Delivery," *Adv. Genet.* 88:111-137, 2014.

Whitehead et al., "Synergistic Silencing: Combinations of Lipid-like Materials for Efficacious siRNA Delivery," *Molecular Therapy* 19(9):1688-1694, 2011.

Wilson et al., "The Combination of Stabilized Plasmid Lipid Particles and Lipid Nanoparticle Encapsulated CpG Containing Oligodeoxynucleotides as Systemic Genetic Vaccine," *The Journal of Gene Medicine* 11(1):14-25, 2009.

Xue et al., "Lipid-Based Nanocarriers for RNA Delivery," *Current Pharmaceutical Design* 21:3140-3147, 2015.

Yin et al., "Therapeutic Genome Editing By Combined Viral and Non-Viral Delivery of CRISPR System Components in vivo," *Nat. Biotech* 34(3):328, 2016.

Yoshimura et al., "Solution Properties of Tadpole-type Cationic Amphiphilic Dendrimers Consisting of an Alkyl Chain, a Quaternary Ammonium, and a Poly(amidoamine) Dendron," *Journal of Oleo Science* 62(4):213-221, 2013.

Yuan et al., "Crystal Structure of *A. aeolicus* Argonaute, A Site-Specific DNA-Guided Endoribonuclease, Provides Insights Into RISC-Mediated mRNA Cleavage," *Molecular Cell* 19:405-419, 2005.

Zanta-Boussif et al., "Validation of a mutated PRE sequence allowing high and sustained transgene expression while abrogating WHV-X protein synthesis: application to the gene therapy of WAS," *Gene Therapy* 16:605-619, 2009.

Zhang et al., "Biodegradable Amino-Ester Nanomaterials for Cas9 mRNA Delivery in Vitro and in Vivo," *ACS Appl. Mater. Interfaces* 9(30):25481-25487, 2017. (15 pages).

Zhang et al., "Lipid-modified spermine derivatives and liposome prepared with said derivatives," Caplus Database, Accession No. 2015:1437089, 2015. (2 pages).

* cited by examiner

1

CATIONIC LIPIDS FOR USE IN LIPID NANOPARTICLES

BACKGROUND

Technical Field

The present disclosure generally relates to novel cationic lipids that can be used in combination with other lipid components, such as neutral lipids, cholesterol and polymer conjugated lipids, to form lipid nanoparticles to facilitate the intracellular delivery of therapeutic agents, such as nucleic acids (e.g., oligonucleotides, messenger RNA), both in vitro and in vivo.

Description of the Related Art

There are many challenges associated with the delivery of nucleic acids to affect a desired response in a biological system. Nucleic acid based therapeutics have enormous potential but there remains a need for more effective delivery of nucleic acids to appropriate sites within a cell or organism in order to realize this potential. Therapeutic nucleic acids include, e.g., messenger RNA (mRNA), antisense oligonucleotides, ribozymes, DNazymes, plasmids, immune stimulating nucleic acids, antagomir, antimir, mimic, supermir, and aptamers. Some nucleic acids, such as mRNA or plasmids, can be used to effect expression of specific cellular products as would be useful in the treatment of, for example, diseases related to a deficiency of a protein or enzyme. The therapeutic applications of translatable nucleotide delivery are extremely broad as constructs can be synthesized to produce any chosen protein sequence, whether or not indigenous to the system. The expression products of the nucleic acid can augment existing levels of protein, replace missing or non-functional versions of a protein, or introduce new protein and associated functionality in a cell or organism.

Some nucleic acids, such as miRNA inhibitors, can be used to effect expression of specific cellular products that are regulated by miRNA as would be useful in the treatment of, for example, diseases related to deficiency of protein or enzyme. The therapeutic applications of miRNA inhibition are extremely broad as constructs can be synthesized to inhibit one or more miRNA that would in turn regulate the expression of mRNA products. The inhibition of endogenous miRNA can augment its downstream target endogenous protein expression and restore proper function in a cell or organism as a means to treat disease associated to a specific miRNA or a group of miRNA.

Other nucleic acids can down-regulate intracellular levels of specific mRNA and, as a result, down-regulate the synthesis of the corresponding proteins through processes such as RNA interference (RNAi) or complementary binding of antisense RNA. The therapeutic applications of antisense oligonucleotide and RNAi are also extremely broad, since oligonucleotide constructs can be synthesized with any nucleotide sequence directed against a target mRNA. Targets may include mRNAs from normal cells, mRNAs associated with disease-states, such as cancer, and mRNAs of infectious agents, such as viruses. To date, antisense oligonucleotide constructs have shown the ability to specifically down-regulate target proteins through degradation of the cognate mRNA in both in vitro and in vivo models. In addition, antisense oligonucleotide constructs are currently being evaluated in clinical studies.

However, two problems currently face the use of oligonucleotides in therapeutic contexts. First, free RNAs are

2

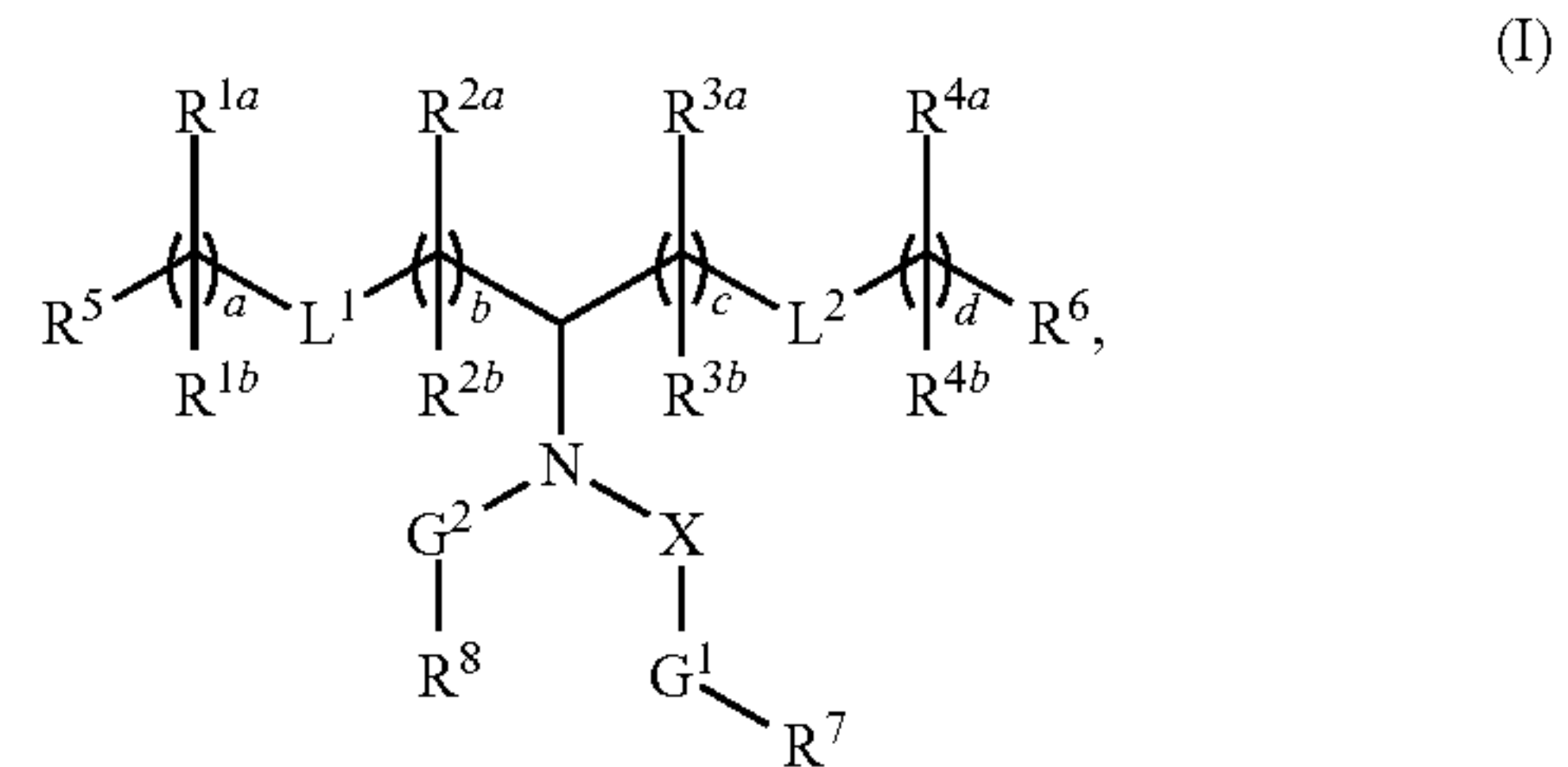
susceptible to nuclease digestion in plasma. Second, free RNAs have limited ability to gain access to the intracellular compartment where the relevant translation machinery resides. Lipid nanoparticles formed from cationic lipids with other lipid components, such as neutral lipids, cholesterol, PEG, PEGylated lipids, and oligonucleotides have been used to block degradation of the RNAs in plasma and facilitate the cellular uptake of the oligonucleotides.

There remains a need for improved cationic lipids and lipid nanoparticles for the delivery of oligonucleotides. Preferably, these lipid nanoparticles would provide optimal drug:lipid ratios, protect the nucleic acid from degradation and clearance in serum, be suitable for systemic or local delivery, and provide intracellular delivery of the nucleic acid. In addition, these lipid-nucleic acid particles should be well-tolerated and provide an adequate therapeutic index, such that patient treatment at an effective dose of the nucleic acid is not associated with unacceptable toxicity and/or risk to the patient. The present disclosure provides these and related advantages.

BRIEF SUMMARY

In brief, the present disclosure provides lipid compounds, including stereoisomers, pharmaceutically acceptable salts or tautomers thereof, which can be used alone or in combination with other lipid components such as neutral lipids, charged lipids, steroids (including for example, all sterols) and/or their analogs, and/or polymer conjugated lipids to form lipid nanoparticles for the delivery of therapeutic agents. In some instances, the lipid nanoparticles are used to deliver nucleic acids such as antisense and/or messenger RNA. Methods for use of such lipid nanoparticles for treatment or prevention (e.g., vaccination) of various diseases or conditions, such as those caused by infectious entities and/or insufficiency of a protein, are also provided.

In one embodiment, compounds having the following structure (I) are provided:



or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein a, b, c, d, G¹, G², L¹, L², R^{1a}, R^{1b}, R^{2a}, R^{2b}, R^{3a}, R^{3b}, R^{4a}, R^{4b}, R⁵, R⁶, R⁷, R⁸ and X are as defined herein.

Lipid nanoparticles (LNPs) comprising one or more of the compounds of structure (I) and a therapeutic agent, and pharmaceutical compositions comprising the same, are also provided. In some embodiments, the nanoparticles further comprise one or more components selected from neutral lipids, charged lipids, steroids, and polymer conjugated lipids. Such LNPs are useful for delivery of the therapeutic agent, for example for treatment of a disease or vaccination against a viral pathogen.

These and other aspects of the disclosure will be apparent upon reference to the following detailed description.

DETAILED DESCRIPTION

In the following description, certain specific details are set forth in order to provide a thorough understanding of various embodiments of the disclosure. However, one skilled in the art will understand that the disclosure may be practiced without these details.

The present disclosure is based, in part, upon the discovery of novel cationic (amino) lipids that provide advantages when used in lipid nanoparticles for the in vivo delivery of an active or therapeutic agent such as a nucleic acid into a cell of a mammal. In particular, embodiments of the present disclosure provide nucleic acid-lipid nanoparticle compositions comprising one or more of the novel cationic lipids described herein that provide increased activity of the nucleic acid and improved tolerability of the compositions in vivo, resulting in a significant increase in the therapeutic index as compared to nucleic acid-lipid nanoparticle compositions previously described. In other embodiments, the disclosed lipids, and lipid nanoparticles comprising the same, have increased safety and/or tolerability when used for delivery of active agents, such as nucleic acids.

In particular embodiments, the present disclosure provides novel cationic lipids that enable the formulation of improved compositions for the in vitro and in vivo delivery of mRNA and/or other oligonucleotides. In some embodiments, these improved lipid nanoparticle compositions are useful for expression of protein encoded by mRNA. In other embodiments, these improved lipid nanoparticles compositions are useful for upregulation of endogenous protein expression by delivering miRNA inhibitors targeting one specific miRNA or a group of miRNA regulating one target mRNA or several mRNA. In other embodiments, these improved lipid nanoparticle compositions are useful for down-regulating (e.g., silencing) the protein levels and/or mRNA levels of target genes. In some other embodiments, the lipid nanoparticles are also useful for delivery of mRNA and plasmids for expression of transgenes. In yet other embodiments, the lipid nanoparticle compositions are useful for inducing a pharmacological effect resulting from expression of a protein, e.g., increased production of red blood cells through the delivery of a suitable erythropoietin mRNA, or protection against infection through delivery of mRNA encoding for a suitable antigen or antibody.

The lipid nanoparticles and compositions of embodiments of the present disclosure may be used for a variety of purposes, including the delivery of encapsulated or associated (e.g., complexed) therapeutic agents such as nucleic acids to cells, both in vitro and in vivo. Accordingly, embodiments of the present disclosure provide methods of treating or preventing diseases or disorders in a subject in need thereof by contacting the subject with a lipid nanoparticle that encapsulates or is associated with a suitable therapeutic agent, wherein the lipid nanoparticle comprises one or more of the novel cationic lipids described herein.

As described herein, embodiments of the lipid nanoparticles of the present disclosure are particularly useful for the delivery of nucleic acids, including, e.g., mRNA, antisense oligonucleotide, plasmid DNA, microRNA (miRNA), miRNA inhibitors (antagomirs/antimirs), messenger-RNA-interfering complementary RNA (micRNA), DNA, multivalent RNA, dicer substrate RNA, complementary DNA (cDNA), etc. Therefore, the lipid nanoparticles and compositions of certain embodiments of the present disclosure may be used to induce expression of a desired protein both in vitro and in vivo by contacting cells with a lipid nanoparticle comprising one or more novel cationic lipids described

herein, wherein the lipid nanoparticle encapsulates or is associated with a nucleic acid that is expressed to produce the desired protein (e.g., a messenger RNA or plasmid encoding the desired protein) or inhibit processes that terminate expression of mRNA (e.g., miRNA inhibitors). In certain embodiments, the protein expressed by the nucleic acid is an antigen, and the LNPs thus induce an immune response (e.g., vaccination). Alternatively, the lipid nanoparticles and compositions of embodiments of the present disclosure may be used to decrease the expression of target genes and proteins both in vitro and in vivo by contacting cells with a lipid nanoparticle comprising one or more novel cationic lipids described herein, wherein the lipid nanoparticle encapsulates or is associated with a nucleic acid that reduces target gene expression (e.g., an antisense oligonucleotide or small interfering RNA (siRNA)). The lipid nanoparticles and compositions of embodiments of the present disclosure may also be used for co-delivery of different nucleic acids (e.g., mRNA and plasmid DNA) separately or in combination, such as may be useful to provide an effect requiring co-localization of different nucleic acids (e.g., mRNA encoding for a suitable gene modifying enzyme and DNA segment(s) for incorporation into the host genome).

Nucleic acids for use with embodiments of this disclosure may be prepared according to any available technique. For mRNA, the primary methodology of preparation is, but not limited to, enzymatic synthesis (also termed in vitro transcription) which currently represents the most efficient method to produce long sequence-specific mRNA. In vitro transcription describes a process of template-directed synthesis of RNA molecules from an engineered DNA template comprised of an upstream bacteriophage promoter sequence (e.g., including but not limited to that from the T7, T3 and SP6 coliphage) linked to a downstream sequence encoding the gene of interest. Template DNA can be prepared for in vitro transcription from a number of sources with appropriate techniques which are well known in the art including, but not limited to, plasmid DNA and polymerase chain reaction amplification (see Linpinsel, J. L. and Conn, G. L., General protocols for preparation of plasmid DNA template and Bowman, J. C., Azizi, B., Lenz, T. K., Ray, P., and Williams, L. D. in RNA in vitro transcription and RNA purification by denaturing PAGE in Recombinant and in vitro RNA syntheses Methods v. 941 Conn G. L. (ed), New York, N.Y. Humana Press, 2012).

Transcription of the RNA occurs in vitro using the linearized DNA template in the presence of the corresponding RNA polymerase and adenosine, guanosine, uridine, and cytidine ribonucleoside triphosphates (rNTPs) under conditions that support polymerase activity while minimizing potential degradation of the resultant mRNA transcripts. In vitro transcription can be performed using a variety of commercially available kits including, but not limited to RiboMax Large Scale RNA Production System (Promega), MegaScript Transcription kits (Life Technologies), as well as with commercially available reagents including RNA polymerases and rNTPs. The methodology for in vitro transcription of mRNA is well known in the art. (see, e.g., Losick, R., 1972, In vitro transcription, *Ann Rev Biochem* v. 41 409-46; Kamakaka, R. T. and Kraus, W. L. 2001. In Vitro Transcription. *Current Protocols in Cell Biology*. 2:11.6: 11.6.1-11.6.17; Beckert, B. And Masquida, B., (2010) Synthesis of RNA by In Vitro Transcription in *RNA in Methods in Molecular Biology* v. 703 (Neilson, H. Ed), New York, N.Y. Humana Press, 2010; Brunelle, J. L. and Green, R., 2013, Chapter Five—In vitro transcription from plasmid or

PCR-amplified DNA, *Methods in Enzymology* v. 530, 101-114; all of which are incorporated herein by reference).

The desired *in vitro* transcribed mRNA is then purified from the undesired components of the transcription or associated reactions (including unincorporated rNTPs, protein enzyme, salts, short RNA oligos, etc.). Techniques for the isolation of the mRNA transcripts are well known in the art. Well known procedures include phenol/chloroform extraction or precipitation with either alcohol (ethanol, isopropanol) in the presence of monovalent cations or lithium chloride. Additional, non-limiting examples of purification procedures which can be used include size exclusion chromatography (Lukaysky, P. J. and Puglisi, J. D., 2004, Large-scale preparation and purification of polyacrylamide-free RNA oligonucleotides, *RNA* v. 10, 889-893), silica-based affinity chromatography and polyacrylamide gel electrophoresis (Bowman, J. C., Azizi, B., Lenz, T. K., Ray, P., and Williams, L. D. in *RNA in vitro transcription and RNA purification by denaturing PAGE in Recombinant and in vitro RNA syntheses Methods* v. 941 Conn G. L. (ed), New York, N.Y. Humana Press, 2012). Purification can be performed using a variety of commercially available kits including, but not limited to SV Total Isolation System (Promega) and In Vitro Transcription Cleanup and Concentration Kit (Norgen Biotek).

Furthermore, while reverse transcription can yield large quantities of mRNA, the products can contain a number of aberrant RNA impurities associated with undesired polymerase activity which may need to be removed from the full-length mRNA preparation. These include short RNAs that result from abortive transcription initiation as well as double-stranded RNA (dsRNA) generated by RNA-dependent RNA polymerase activity, RNA-primed transcription from RNA templates and self-complementary 3' extension. It has been demonstrated that these contaminants with dsRNA structures can lead to undesired immunostimulatory activity through interaction with various innate immune sensors in eukaryotic cells that function to recognize specific nucleic acid structures and induce potent immune responses. This in turn, can dramatically reduce mRNA translation since protein synthesis is reduced during the innate cellular immune response. Therefore, additional techniques to remove these dsRNA contaminants have been developed and are known in the art including but not limited to scaleable HPLC purification (see, e.g., Kariko, K., Muramatsu, H., Ludwig, J. And Weissman, D., 2011, Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA, *Nucl Acid Res*, v. 39 e142; Weissman, D., Pardi, N., Muramatsu, H., and Kariko, K., HPLC Purification of *in vitro* transcribed long RNA in *Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology* v. 969 (Rabinovich, P. H. Ed), 2013). HPLC purified mRNA has been reported to be translated at much greater levels, particularly in primary cells and *in vivo*.

A significant variety of modifications have been described in the art which are used to alter specific properties of *in vitro* transcribed mRNA, and improve its utility. These include, but are not limited to modifications to the 5' and 3' termini of the mRNA. Endogenous eukaryotic mRNA typically contain a cap structure on the 5'-end of a mature molecule which plays an important role in mediating binding of the mRNA Cap Binding Protein (CBP), which is in turn responsible for enhancing mRNA stability in the cell and efficiency of mRNA translation. Therefore, highest levels of protein expression are achieved with capped

mRNA transcripts. The 5'-cap contains a 5'-5'-triphosphate linkage between the 5'-most nucleotide and guanine nucleotide. The conjugated guanine nucleotide is methylated at the N7 position. Additional modifications include methylation of the ultimate and penultimate most 5'-nucleotides on the 2'-hydroxyl group.

Multiple distinct cap structures can be used to generate the 5'-cap of *in vitro* transcribed synthetic mRNA. 5'-capping of synthetic mRNA can be performed co-transcriptionally with chemical cap analogs (i.e., capping during *in vitro* transcription). For example, the Anti-Reverse Cap Analog (ARCA) cap contains a 5'-5'-triphosphate guanine-guanine linkage where one guanine contains an N7 methyl group as well as a 3'-O-methyl group. However, up to 20% of transcripts remain uncapped during this co-transcriptional process and the synthetic cap analog is not identical to the 5'-cap structure of an authentic cellular mRNA, potentially reducing translatability and cellular stability. Alternatively, synthetic mRNA molecules may also be enzymatically capped post-transcriptionally. These may generate a more authentic 5'-cap structure that more closely mimics, either structurally or functionally, the endogenous 5'-cap which have enhanced binding of cap binding proteins, increased half-life and reduced susceptibility to 5' endonucleases and/or reduced 5' decapping. Numerous synthetic 5'-cap analogs have been developed and are known in the art to enhance mRNA stability and translatability (see, e.g., Grudzien-Nogalska, E., Kowalska, J., Su, W., Kuhn, A. N., Slepnev, S. V., Darynkiewicz, E., Sahin, U., Jemielity, J., and Rhoads, R. E., Synthetic mRNAs with superior translation and stability properties in *Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology* v. 969 (Rabinovich, P. H. Ed), 2013).

On the 3'-terminus, a long chain of adenine nucleotides (poly-A tail) is normally added to mRNA molecules during RNA processing. Immediately after transcription, the 3' end of the transcript is cleaved to free a 3' hydroxyl to which poly-A polymerase adds a chain of adenine nucleotides to the RNA in a process called polyadenylation. The poly-A tail has been extensively shown to enhance both translational efficiency and stability of mRNA (see Bernstein, P. and Ross, J., 1989, Poly (A), poly (A) binding protein and the regulation of mRNA stability, *Trends Bio Sci* v. 14 373-377; Guhaniyogi, J. And Brewer, G., 2001, Regulation of mRNA stability in mammalian cells, *Gene*, v. 265, 11-23; Dreyfus, M. And Regnier, P., 2002, The poly (A) tail of mRNAs: Bodyguard in eukaryotes, scavenger in bacteria, *Cell*, v. 111, 611-613).

Poly (A) tailing of *in vitro* transcribed mRNA can be achieved using various approaches including, but not limited to, cloning of a poly (T) tract into the DNA template or by post-transcriptional addition using Poly (A) polymerase. The first case allows *in vitro* transcription of mRNA with poly (A) tails of defined length, depending on the size of the poly (T) tract, but requires additional manipulation of the template. The latter case involves the enzymatic addition of a poly (A) tail to *in vitro* transcribed mRNA using poly (A) polymerase which catalyzes the incorporation of adenine residues onto the 3' termini of RNA, requiring no additional manipulation of the DNA template, but results in mRNA with poly(A) tails of heterogeneous length. 5'-capping and 3'-poly (A) tailing can be performed using a variety of commercially available kits including, but not limited to Poly (A) Polymerase Tailing kit (EpiCenter), mMACHINE mMESSAGE T7 Ultra kit and Poly (A) Tailing kit (Life Technologies) as well as with commercially available reagents, various ARCA caps, Poly (A) polymerase, etc.

In addition to 5' cap and 3' poly adenylation, other modifications of the in vitro transcripts have been reported to provide benefits as related to efficiency of translation and stability. It is well known in the art that pathogenic DNA and RNA can be recognized by a variety of sensors within eukaryotes and trigger potent innate immune responses. The ability to discriminate between pathogenic and self DNA and RNA has been shown to be based, at least in part, on structure and nucleoside modifications since most nucleic acids from natural sources contain modified nucleosides. In contrast, in vitro synthesized RNA lacks these modifications, thus rendering it immunostimulatory which in turn can inhibit effective mRNA translation as outlined above. The introduction of modified nucleosides into in vitro transcribed mRNA can be used to prevent recognition and activation of RNA sensors, thus mitigating this undesired immunostimulatory activity and enhancing translation capacity (see, e.g., Kariko, K. And Weissman, D. 2007, Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development, *Curr Opin Drug Discov Devel*, v. 10 523-532; Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology* v. 969 (Rabinovich, P. H. Ed), 2013; Kariko, K., Muramatsu, H., Welsh, F. A., Ludwig, J., Kato, H., Akira, S., Weissman, D., 2008, Incorporation of Pseudouridine Into mRNA Yields Superior Non-immunogenic Vector With Increased Translational Capacity and Biological Stability, *Mol Ther* v. 16, 1833-1840). The modified nucleosides and nucleotides used in the synthesis of modified RNAs can be prepared monitored and utilized using general methods and procedures known in the art. A large variety of nucleoside modifications are available that may be incorporated alone or in combination with other modified nucleosides to some extent into the in vitro transcribed mRNA (see, e.g., US 2012/0251618). In vitro synthesis of nucleoside-modified mRNA has been reported to have reduced ability to activate immune sensors with a concomitant enhanced translational capacity.

Other components of mRNA which can be modified to provide benefit in terms of translatability and stability include the 5' and 3' untranslated regions (UTR). Optimization of the UTRs (favorable 5' and 3' UTRs can be obtained from cellular or viral RNAs), either both or independently, have been shown to increase mRNA stability and translational efficiency of in vitro transcribed mRNA (see, e.g., Pardi, N., Muramatsu, H., Weissman, D., Kariko, K., *In vitro transcription of long RNA containing modified nucleosides in Synthetic Messenger RNA and Cell Metabolism Modulation in Methods in Molecular Biology* v. 969 (Rabinovich, P. H. Ed), 2013).

In addition to mRNA, other nucleic acid payloads may be used for this disclosure. For oligonucleotides, methods of preparation include but are not limited to chemical synthesis and enzymatic, chemical cleavage of a longer precursor, in vitro transcription as described above, etc. Methods of synthesizing DNA and RNA nucleotides are widely used and well known in the art (see, e.g., Gait, M. J. (ed.) *Oligonucleotide synthesis: a practical approach*, Oxford [Oxfordshire], Washington, D.C.: IRL Press, 1984; and Herdewijn, P. (ed.) *Oligonucleotide synthesis: methods and applications*, *Methods in Molecular Biology*, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

For plasmid DNA, preparation for use with embodiments of this disclosure commonly utilizes, but is not limited to,

expansion and isolation of the plasmid DNA in vitro in a liquid culture of bacteria containing the plasmid of interest. The presence of a gene in the plasmid of interest that encodes resistance to a particular antibiotic (penicillin, kanamycin, etc.) allows those bacteria containing the plasmid of interest to selectively grow in antibiotic-containing cultures. Methods of isolating plasmid DNA are widely used and well known in the art (see, e.g., Heilig, J., Elbing, K. L. and Brent, R., (2001), *Large-Scale Preparation of Plasmid DNA*, *Current Protocols in Molecular Biology*, 41:11:1.7: 1.7.1-1.7.16; Rozkov, A., Larsson, B., Gillström, S., Björnstedt, R. and Schmidt, S. R., (2008), *Large-scale production of endotoxin-free plasmids for transient expression in mammalian cell culture*, *Biotechnol. Bioeng.*, 99: 557-566; and U.S. Pat. No. 6,197,553 B1). Plasmid isolation can be performed using a variety of commercially available kits including, but not limited to Plasmid Plus (Qiagen), GenJET plasmid MaxiPrep (Thermo), and PureYield MaxiPrep (Promega) kits as well as with commercially available reagents.

Various exemplary embodiments of the cationic lipids of the present disclosure, lipid nanoparticles and compositions comprising the same, and their use to deliver active (e.g., therapeutic agents), such as nucleic acids, to modulate gene and protein expression, are described in further detail below.

As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

Unless the context requires otherwise, throughout the present specification and claims, the word “comprise” and variations thereof, such as, “comprises” and “comprising” are to be construed in an open and inclusive sense, that is, as “including, but not limited to.”

Reference throughout this specification to “one embodiment” or “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present disclosure. Thus, the appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this disclosure belongs. As used in the specification and claims, the singular form “a,” “an” and “the” include plural references unless the context clearly dictates otherwise.

The phrase “induce expression of a desired protein” refers to the ability of a nucleic acid to increase expression of the desired protein. To examine the extent of protein expression, a test sample (e.g., a sample of cells in culture expressing the desired protein) or a test mammal (e.g., a mammal such as a human or an animal) model such as a rodent (e.g., mouse) or a non-human primate (e.g., monkey) model is contacted with a nucleic acid (e.g., nucleic acid in combination with a lipid of the present disclosure). Expression of the desired protein in the test sample or test animal is compared to expression of the desired protein in a control sample (e.g., a sample of cells in culture expressing the desired protein) or a control mammal (e.g., a mammal such as a human or an animal) model such as a rodent (e.g., mouse) or non-human primate (e.g., monkey) model that is not contacted with or administered the nucleic acid. When the desired protein is present in a control sample or a control mammal, the expression of a desired protein in a control sample or a control mammal may be assigned a value of 1.0. In particular embodiments, inducing expression of a desired protein is

achieved when the ratio of desired protein expression in the test sample or the test mammal to the level of desired protein expression in the control sample or the control mammal is greater than 1, for example, about 1.1, 1.5, 2.0, 5.0 or 10.0. When a desired protein is not present in a control sample or a control mammal, inducing expression of a desired protein is achieved when any measurable level of the desired protein in the test sample or the test mammal is detected. One of ordinary skill in the art will understand appropriate assays to determine the level of protein expression in a sample, for example dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, and phenotypic assays, or assays based on reporter proteins that can produce fluorescence or luminescence under appropriate conditions.

The phrase “inhibiting expression of a target gene” refers to the ability of a nucleic acid to silence, reduce, or inhibit the expression of a target gene. To examine the extent of gene silencing, a test sample (e.g., a sample of cells in culture expressing the target gene) or a test mammal (e.g., a mammal such as a human or an animal) model such as a rodent (e.g., mouse) or a non-human primate (e.g., monkey) model is contacted with a nucleic acid that silences, reduces, or inhibits expression of the target gene. Expression of the target gene in the test sample or test animal is compared to expression of the target gene in a control sample (e.g., a sample of cells in culture expressing the target gene) or a control mammal (e.g., a mammal such as a human or an animal) model such as a rodent (e.g., mouse) or non-human primate (e.g., monkey) model that is not contacted with or administered the nucleic acid. The expression of the target gene in a control sample or a control mammal may be assigned a value of 100%. In particular embodiments, silencing, inhibition, or reduction of expression of a target gene is achieved when the level of target gene expression in the test sample or the test mammal relative to the level of target gene expression in the control sample or the control mammal is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. In other words, the nucleic acids are capable of silencing, reducing, or inhibiting the expression of a target gene by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% in a test sample or a test mammal relative to the level of target gene expression in a control sample or a control mammal not contacted with or administered the nucleic acid. Suitable assays for determining the level of target gene expression include, without limitation, examination of protein or mRNA levels using techniques known to those of skill in the art, such as, e.g., dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, as well as phenotypic assays known to those of skill in the art.

An “effective amount” or “therapeutically effective amount” of an active agent or therapeutic agent such as a therapeutic nucleic acid is an amount sufficient to produce the desired effect, e.g., an increase or inhibition of expression of a target sequence in comparison to the normal expression level detected in the absence of the nucleic acid. An increase in expression of a target sequence is achieved when any measurable level is detected in the case of an expression product that is not present in the absence of the nucleic acid. In the case where the expression product is present at some level prior to contact with the nucleic acid, an increase in expression is achieved when the fold increase in value obtained with a nucleic acid such as mRNA relative to control is about 1.05, 1.1, 1.2, 1.3, 1.4, 1.5, 1.75,

2, 2.5, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 40, 50, 75, 100, 250, 500, 750, 1000, 5000, 10000, or greater. Inhibition of expression of a target gene or target sequence is achieved when the value obtained with a nucleic acid such as antisense oligonucleotide relative to the control is about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, or 0%. Suitable assays for measuring expression of a target gene or target sequence include, e.g., examination of protein or RNA levels using techniques known to those of skill in the art such as dot blots, northern blots, in situ hybridization, ELISA, immunoprecipitation, enzyme function, fluorescence, or luminescence of suitable reporter proteins, as well as phenotypic assays known to those of skill in the art.

The term “nucleic acid” as used herein refers to a polymer containing at least two deoxyribonucleotides or ribonucleotides in either single- or double-stranded form and includes DNA, RNA, and hybrids thereof. DNA may be in the form of antisense molecules, plasmid DNA, cDNA, PCR products, or vectors. RNA may be in the form of small hairpin RNA (shRNA), messenger RNA (mRNA), antisense RNA, miRNA, micRNA, multivalent RNA, dicer substrate RNA or viral RNA (vRNA), and combinations thereof. Nucleic acids include nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, and which have similar binding properties as the reference nucleic acid. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2'-O-methyl ribonucleotides, and peptide-nucleic acids (PNAs). Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, single nucleotide polymorphisms, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., *Nucleic Acid Res.*, 19:5081 (1991); Ohtsuka et al., *J. Biol. Chem.*, 260:2605-2608 (1985); Rossolini et al., *Mol. Cell. Probes*, 8:91-98 (1994)). “Nucleotides” contain a sugar deoxyribose (DNA) or ribose (RNA), a base, and a phosphate group. Nucleotides are linked together through the phosphate groups. “Bases” include purines and pyrimidines, which further include natural compounds adenine, thymine, guanine, cytosine, uracil, inosine, and natural analogs, and synthetic derivatives of purines and pyrimidines, which include, but are not limited to, modifications which place new reactive groups such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides.

The term “gene” refers to a nucleic acid (e.g., DNA or RNA) sequence that comprises partial length or entire length coding sequences necessary for the production of a polypeptide or precursor polypeptide.

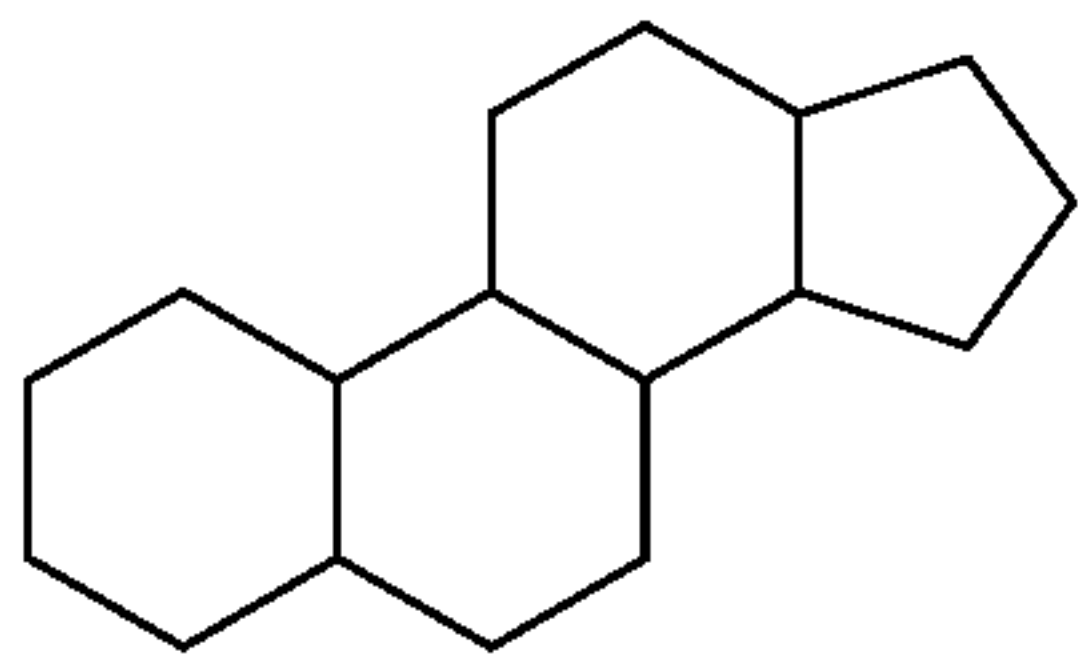
“Gene product,” as used herein, refers to a product of a gene such as an RNA transcript or a polypeptide.

The term “lipid” refers to a group of organic compounds that include, but are not limited to, esters of fatty acids and are generally characterized by being poorly soluble in water, but soluble in many organic solvents. They are usually divided into at least three classes: (1) “simple lipids,” which include fats and oils as well as waxes; (2) “compound

11

lipids,” which include phospholipids and glycolipids; and (3) “derived lipids” such as steroids.

A “steroid” is a compound comprising the following carbon skeleton:



Non-limiting examples of steroids include cholesterol, and the like.

A “cationic lipid” refers to a lipid capable of being positively charged. Exemplary cationic lipids include one or more amine group(s) which bear the positive charge.

Preferred cationic lipids are ionizable such that they can exist in a positively charged or neutral form depending on pH. The ionization of the cationic lipid affects the surface charge of the lipid nanoparticle under different pH conditions. This charge state can influence plasma protein absorption, blood clearance, and tissue distribution (Semple, S. C., et al., *Adv. Drug Deliv Rev* 32:3-17 (1998)) as well as the ability to form endosomolytic non-bilayer structures (Hafez, I. M., et al., *Gene Ther* 8:1188-1196 (2001)) critical to the intracellular delivery of nucleic acids.

The term “polymer conjugated lipid” refers to a molecule comprising both a lipid portion and a polymer portion. An example of a polymer conjugated lipid is a pegylated lipid. The term “pegylated lipid” refers to a molecule comprising both a lipid portion and a polyethylene glycol portion. Pegylated lipids are known in the art and include 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG) and the like.

The term “neutral lipid” refers to any of a number of lipid species that exist either in an uncharged or neutral zwitterionic form at a selected pH. At physiological pH, such lipids include, but are not limited to, phosphatidylcholines such as 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), phosphatidylethanolamines such as 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), sphingomyelins (SM), ceramides, steroids such as sterols and their derivatives. Neutral lipids may be synthetic or naturally derived.

The term “charged lipid” refers to any of a number of lipid species that exist in either a positively charged or negatively charged form independent of the pH within a useful physiological range, e.g., pH ~3 to pH ~9. Charged lipids may be synthetic or naturally derived. Examples of charged lipids include phosphatidylserines, phosphatidic acids, phosphatidylglycerols, phosphatidylinositols, sterol hemi succinates, dialkyl trimethylammonium-propanes, (e.g., DOTAP, DOTMA), dialkyl dimethylaminopropanes, ethyl phosphocholines, dimethylaminoethane carbamoyl sterols (e.g., DC-Chol).

The term “lipid nanoparticle” refers to particles having at least one dimension on the order of nanometers (e.g., 1-1,000 nm) which include one or more of the compounds of structure (I) or other specified cationic lipids. In some embodiments, lipid nanoparticles comprising the disclosed

12

cationic lipids (e.g., compounds of structure (I)) are included in a formulation that can be used to deliver an active agent or therapeutic agent, such as a nucleic acid (e.g., mRNA) to a target site of interest (e.g., cell, tissue, organ, tumor, and the like). In some embodiments, the lipid nanoparticles comprise a compound of structure (I) and a nucleic acid. Such lipid nanoparticles typically comprise a compound of structure (I) and one or more excipient selected from neutral lipids, charged lipids, steroids, and polymer conjugated lipids. In some embodiments, the active agent or therapeutic agent, such as a nucleic acid, may be encapsulated in the lipid portion of the lipid nanoparticle or an aqueous space enveloped by some or all of the lipid portion of the lipid nanoparticle, thereby protecting it from enzymatic degradation or other undesirable effects induced by the mechanisms of the host organism or cells, e.g., an adverse immune response.

In various embodiments, the lipid nanoparticles have a mean diameter of from about 30 nm to about 150 nm, from about 40 nm to about 150 nm, from about 50 nm to about 150 nm, from about 60 nm to about 130 nm, from about 70 nm to about 110 nm, from about 70 nm to about 100 nm, from about 80 nm to about 100 nm, from about 90 nm to about 100 nm, from about 70 to about 90 nm, from about 80 nm to about 90 nm, from about 70 nm to about 80 nm, or about 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 55 nm, 60 nm, 65 nm, 70 nm, 75 nm, 80 nm, 85 nm, 90 nm, 95 nm, 100 nm, 105 nm, 110 nm, 115 nm, 120 nm, 125 nm, 130 nm, 135 nm, 140 nm, 145 nm, or 150 nm. In some embodiments, the lipid nanoparticles are substantially non-toxic. In certain embodiments, nucleic acids, when present in the lipid nanoparticles, are resistant in aqueous solution to degradation with a nuclease. Lipid nanoparticles comprising nucleic acids and their method of preparation are disclosed in, e.g., U.S. Patent Pub. Nos. 2004/0142025, 2007/0042031 and PCT Pub. Nos. WO 2013/016058 and WO 2013/086373, the full disclosures of which are herein incorporated by reference in their entirety for all purposes.

As used herein, “lipid encapsulated” refers to a lipid nanoparticle that provides an active agent or therapeutic agent, such as a nucleic acid (e.g., mRNA), with full encapsulation, partial encapsulation, or both. In an embodiment, the nucleic acid (e.g., mRNA) is fully encapsulated in the lipid nanoparticle.

As used herein, the term “aqueous solution” refers to a composition comprising water.

“Serum-stable” in relation to nucleic acid-lipid nanoparticles means that the nucleotide is not significantly degraded after exposure to a serum or nuclease assay that would significantly degrade free DNA or RNA. Suitable assays include, for example, a standard serum assay, a DNase assay, or an RNase assay.

“Systemic delivery,” as used herein, refers to delivery of a therapeutic product that can result in a broad exposure of an active agent within an organism. Some techniques of administration can lead to the systemic delivery of certain agents, but not others. Systemic delivery means that a useful, preferably therapeutic, amount of an agent is exposed to most parts of the body. Systemic delivery of lipid nanoparticles can be by any means known in the art including, for example, intravenous, intraarterial, subcutaneous, and intraperitoneal delivery. In some embodiments, systemic delivery of lipid nanoparticles is by intravenous delivery.

“Local delivery,” as used herein, refers to delivery of an active agent directly to a target site within an organism. For example, an agent can be locally delivered by direct injection into a disease site such as a tumor, other target site such

as a site of inflammation, or a target organ such as the liver, heart, pancreas, kidney, and the like. Local delivery can also include topical applications or localized injection techniques such as intramuscular, subcutaneous, or intradermal injection. Local delivery does not preclude a systemic pharmacological effect.

“Alkyl” refers to a straight or branched hydrocarbon chain radical consisting solely of carbon and hydrogen atoms, which is saturated, and having, for example, from one to twenty-four carbon atoms (C_1 - C_{24} alkyl), four to twenty carbon atoms (C_4 - C_{20} alkyl), six to sixteen carbon atoms (C_6 - C_{16} alkyl), six to nine carbon atoms (C_6 - C_9 alkyl), one to fifteen carbon atoms (C_1 - C_{15} alkyl), one to twelve carbon atoms (C_1 - C_{12} alkyl), one to eight carbon atoms (C_1 - C_8 alkyl) or one to six carbon atoms (C_1 - C_6 alkyl) and which is attached to the rest of the molecule by a single bond, e.g., methyl, ethyl, n-propyl, 1-methylethyl (iso propyl), n-butyl, n-pentyl, 1,1-dimethylethyl (t-butyl), 3-methylhexyl, 2-methylhexyl, and the like. Unless stated otherwise specifically in the specification, an alkyl group is substituted or unsubstituted.

“Alkylene” refers to a straight or branched divalent hydrocarbon chain linking the rest of the molecule to a radical group, consisting solely of carbon and hydrogen, which is saturated, and having, for example, from one to twenty-four carbon atoms (C_1 - C_{24} alkylene), one to fifteen carbon atoms (C_1 - C_{15} alkylene), one to twelve carbon atoms (C_1 - C_{12} alkylene), one to eight carbon atoms (C_1 - C_8 alkylene), one to six carbon atoms (C_1 - C_6 alkylene), two to four carbon atoms (C_2 - C_4 alkylene), one to two carbon atoms (C_1 - C_2 alkylene), e.g., methylene, ethylene, propylene, n-butylene, and the like. The alkylene chain is attached to the rest of the molecule through a single bond and to the radical group through a single bond. The points of attachment of the alkylene chain to the rest of the molecule and to the radical group can be through one carbon or any two carbons within the chain. Unless stated otherwise specifically in the specification, an alkylene chain is substituted or unsubstituted.

“Alkene” and “alkenylene” refer to an alkyl and alkylene, respectively, comprising at least one carbon-carbon double bond. Alkenes and alkenylenes include the same number of carbon atoms as alkyl and alkylene as defined above, except that alkenes and alkenylenes must include at least two carbons. Unless stated otherwise specifically in the specification, alkenes and alkenylenes are substituted or unsubstituted.

The term “substituted” used herein means any of the above groups (e.g., alkyl or alkylene) wherein at least one hydrogen atom is replaced by a bond to a non-hydrogen atom such as, but not limited to: a halogen atom such as F, Cl, Br, or I; oxo groups ($=O$); hydroxyl groups ($-OH$); carboxyl groups $-(CO_2H)$; C_1 - C_{12} alkyl groups; $-(C=O)OR'$; $-O(C=O)R'$; $-C(=O)R'$; $-OR'$; $-S(O)_xR'$; $-S-SR'$; $-C(=O)SR'$; $-SC(=O)R'$; $-NR'R'$; $-NRC(=O)R'$; $-C(=O)NR'R'$; $-NRC(=O)NR'R'$; $-OC(=O)NR'R'$; $-NR'C(=O)OR'$; $-NR'S(O)_xNR'R'$; $-NRS(O)_xR'$; and $-S(O)_xNR'R'$, wherein: R' is, at each occurrence, independently H or C_1 - C_{15} alkyl and x is 0, 1 or 2. In some embodiments the substituent is a C_1 - C_{12} alkyl group. In other embodiments, the substituent is a halo group, such as fluoro. In other embodiments, the substituent is an oxo group. In other embodiments, the substituent is a hydroxyl group. In other embodiments, the substituent is an alkoxy group ($-OR$). In other embodiments, the substituent is a carboxyl group. In other embodiments, the substituent is an amine group ($-NR'R'$).

“Optional” or “optionally” (e.g., optionally substituted) means that the subsequently described event or circumstances may or may not occur, and that the description includes instances where said event or circumstance occurs and instances in which it does not. For example, “optionally substituted alkyl” means that the alkyl radical may or may not be substituted and that the description includes both substituted alkyl radicals and alkyl radicals having no substitution.

The disclosure disclosed herein is also meant to encompass all pharmaceutically acceptable compounds of the compound of structure (I) being isotopically-labelled by having one or more atoms replaced by an atom having a different atomic mass or mass number. Examples of isotopes that can be incorporated into the disclosed compounds include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorous, fluorine, chlorine, and iodine, such as 2H , 3H , ^{11}C , ^{13}C , ^{14}C , ^{13}N , ^{15}N , ^{15}O , ^{17}O , ^{18}O , ^{31}P , ^{32}P , ^{35}S , ^{18}F , ^{36}Cl , ^{123}I , and ^{125}I , respectively. These radiolabeled compounds could be useful to help determine or measure the effectiveness of the compounds, by characterizing, for example, the site or mode of action, or binding affinity to pharmacologically important site of action. Certain isotopically-labelled compounds of structure (I), (IA) or (IB), for example, those incorporating a radioactive isotope, are useful in drug and/or substrate tissue distribution studies. The radioactive isotopes tritium, i.e., 3H , and carbon-14, i.e., ^{14}C , are particularly useful for this purpose in view of their ease of incorporation and ready means of detection.

Substitution with heavier isotopes such as deuterium, i.e., 2H , may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased in vivo half-life or reduced dosage requirements, and hence may be preferred in some circumstances.

Substitution with positron emitting isotopes, such as ^{11}C , ^{18}F , ^{15}O and ^{13}N , can be useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy. Isotopically-labeled compounds of structure (I) can generally be prepared by conventional techniques known to those skilled in the art or by processes analogous to those described in the Preparations and Examples as set out below using an appropriate isotopically-labeled reagent in place of the non-labeled reagent previously employed.

“Stable compound” and “stable structure” are meant to indicate a compound that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and formulation into an efficacious therapeutic agent.

“Mammal” includes humans and both domestic animals such as laboratory animals and household pets (e.g., cats, dogs, swine, cattle, sheep, goats, horses, rabbits), and non-domestic animals such as wildlife and the like.

“Pharmaceutically acceptable carrier, diluent or excipient” includes without limitation any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals.

“Pharmaceutically acceptable salt” includes both acid and base addition salts.

“Pharmaceutically acceptable acid addition salt” refers to those salts which retain the biological effectiveness and properties of the free bases, which are not biologically or otherwise undesirable, and which are formed with inorganic acids such as, but are not limited to, hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid

and the like, and organic acids such as, but not limited to, acetic acid, 2,2-dichloroacetic acid, adipic acid, alginic acid, ascorbic acid, aspartic acid, benzenesulfonic acid, benzoic acid, 4-acetamidobenzoic acid, camphoric acid, camphor-10-sulfonic acid, capric acid, caproic acid, caprylic acid, carbonic acid, cinnamic acid, citric acid, cyclamic acid, dodecyl sulfuric acid, ethane-1,2-disulfonic acid, ethane-sulfonic acid, 2-hydroxyethanesulfonic acid, formic acid, fumaric acid, galactaric acid, gentisic acid, glucoheptonic acid, gluconic acid, glucuronic acid, glutamic acid, glutaric acid, 2-oxo-glutaric acid, glycerophosphoric acid, glycolic acid, hippuric acid, isobutyric acid, lactic acid, lactobionic acid, lauric acid, maleic acid, malic acid, malonic acid, mandelic acid, methanesulfonic acid, mucic acid, naphthalene-1,5-disulfonic acid, naphthalene-2-sulfonic acid, 1-hydroxy-2-naphthoic acid, nicotinic acid, oleic acid, orotic acid, oxalic acid, palmitic acid, pamoic acid, propionic acid, pyroglutamic acid, pyruvic acid, salicylic acid, 4-aminosalicylic acid, sebacic acid, stearic acid, succinic acid, tartaric acid, thiocyanic acid, p-toluenesulfonic acid, trifluoroacetic acid, undecylenic acid, and the like.

“Pharmaceutically acceptable base addition salt” refers to those salts which retain the biological effectiveness and properties of the free acids, which are not biologically or otherwise undesirable. These salts are prepared from addition of an inorganic base or an organic base to the free acid. Salts derived from inorganic bases include, but are not limited to, the sodium, potassium, lithium, ammonium, calcium, magnesium, iron, zinc, copper, manganese, aluminum salts and the like. Preferred inorganic salts are the ammonium, sodium, potassium, calcium, and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines and basic ion exchange resins, such as ammonia, isopropylamine, trimethylamine, diethylamine, triethylamine, tripropylamine, diethanolamine, ethanolamine, deanol, 2-dimethylaminoethanol, 2-diethylaminoethanol, dicyclohexylamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, benethamine, benzathine, ethylenediamine, glucosamine, methylglucamine, theobromine, triethanolamine, tromethamine, purines, piperazine, piperidine, N-ethylpiperidine, polyamine resins and the like. Particularly preferred organic bases are isopropylamine, diethylamine, ethanolamine, triethylamine, dicyclohexylamine, choline and caffeine.

Often crystallizations produce a solvate of a compound of the disclosure (i.e., a compound of structure (I)). As used herein, the term “solvate” refers to an aggregate that comprises one or more molecules of a compound of the disclosure with one or more molecules of solvent. The solvent may be water, in which case the solvate may be a hydrate. Alternatively, the solvent may be an organic solvent. Thus, the compounds of the present disclosure may exist as a hydrate, including a monohydrate, dihydrate, hemihydrate, sesquihydrate, trihydrate, tetrahydrate and the like, as well as the corresponding solvated forms. Solvates of compound of the disclosure may be true solvates, while in other cases the compound of the disclosure may merely retain adventitious water or be a mixture of water plus some adventitious solvent.

A “pharmaceutical composition” refers to a formulation of a compound of the disclosure and a medium generally accepted in the art for the delivery of the biologically active compound to mammals, e.g., humans. Such a medium includes all pharmaceutically acceptable carriers, diluents or excipients therefor.

“Effective amount” or “therapeutically effective amount” refers to that amount of a compound of the disclosure which, when administered to a mammal, preferably a human, is sufficient to effect treatment in the mammal, preferably a human. The amount of a lipid nanoparticle of the disclosure which constitutes a “therapeutically effective amount” will vary depending on the compound, the condition and its severity, the manner of administration, and the age of the mammal to be treated, but can be determined routinely by one of ordinary skill in the art having regard to his own knowledge and to this disclosure.

“Treating” or “treatment” as used herein covers the treatment of the disease or condition of interest in a mammal, preferably a human, having the disease or condition of interest, and includes:

- (i) preventing the disease or condition from occurring in a mammal, in particular, when such mammal is predisposed to the condition but has not yet been diagnosed as having it;
- (ii) inhibiting the disease or condition, i.e., arresting its development;
- (iii) relieving the disease or condition, i.e., causing regression of the disease or condition; or
- (iv) relieving the symptoms resulting from the disease or condition, i.e., relieving pain without addressing the underlying disease or condition. As used herein, the terms “disease” and “condition” may be used interchangeably or may be different in that the particular malady or condition may not have a known causative agent (so that etiology has not yet been worked out) and it is therefore not yet recognized as a disease but only as an undesirable condition or syndrome, wherein a more or less specific set of symptoms have been identified by clinicians.

The compounds of the disclosure, or their pharmaceutically acceptable salts may contain one or more stereocenters and may thus give rise to enantiomers, diastereomers, and other stereoisomeric forms that may be defined, in terms of absolute stereochemistry, as (R)- or (S)- or, as (D)- or (L)- for amino acids. The present disclosure is meant to include all such possible isomers, as well as their racemic and optically pure forms. Optically active (+) and (–), (R)- and (S)-, or (D)- and (L)-isomers may be prepared using chiral synthons or chiral reagents, or resolved using conventional techniques, for example, chromatography and fractional crystallization. Conventional techniques for the preparation/isolation of individual enantiomers include chiral synthesis from a suitable optically pure precursor or resolution of the racemate (or the racemate of a salt or derivative) using, for example, chiral high pressure liquid chromatography (HPLC). When the compounds described herein contain olefinic double bonds or other centers of geometric asymmetry, and unless specified otherwise, it is intended that the compounds include both E and Z geometric isomers. Likewise, all tautomeric forms are also intended to be included.

A “stereoisomer” refers to a compound made up of the same atoms bonded by the same bonds but having different three-dimensional structures, which are not interchangeable. The present disclosure contemplates various stereoisomers and mixtures thereof and includes “enantiomers,” which refers to two stereoisomers whose molecules are non-superimposable mirror images of one another.

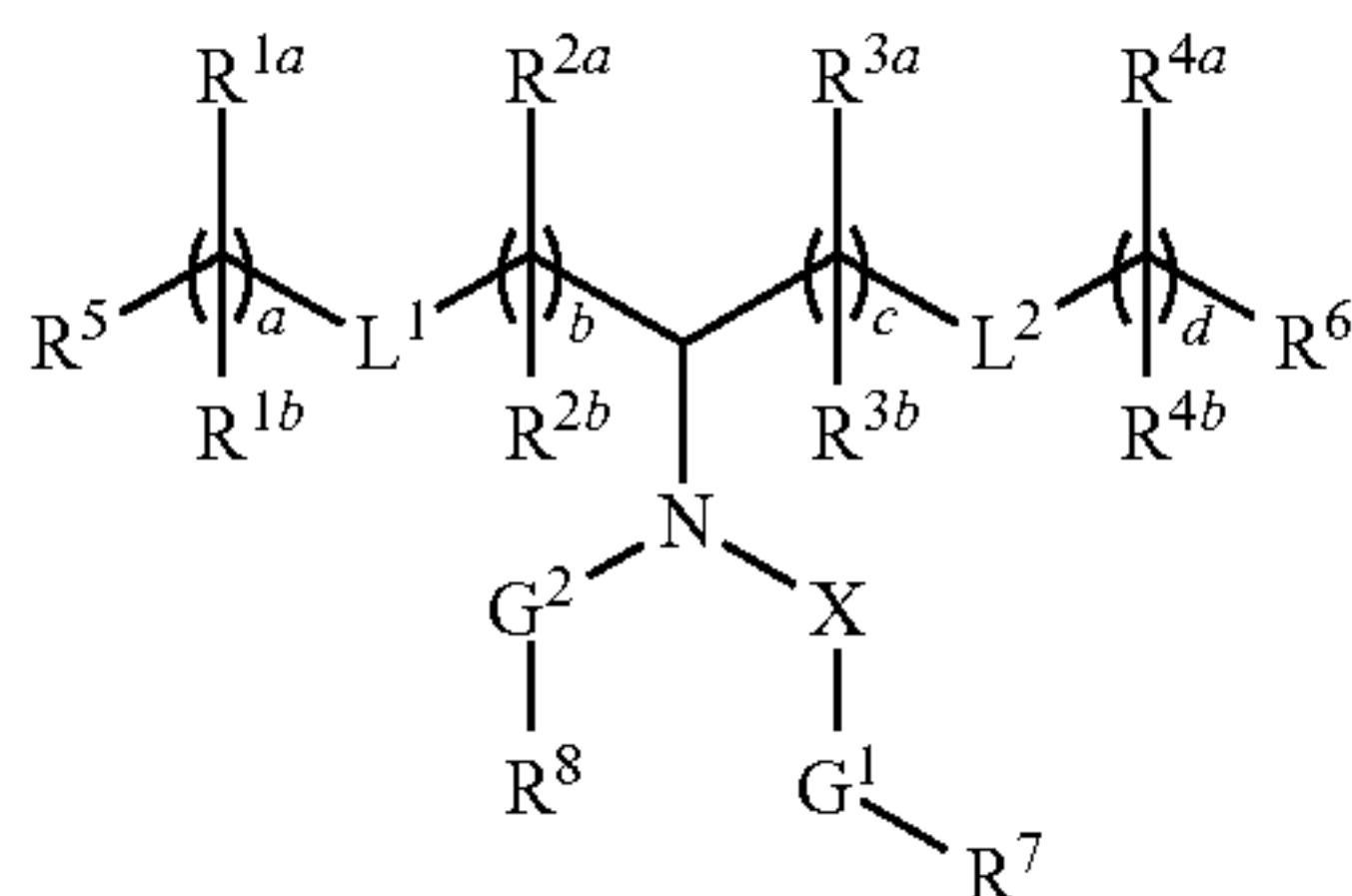
A “tautomer” refers to a proton shift from one atom of a molecule to another atom of the same molecule. The present disclosure includes tautomers of any said compounds.

17

Compounds

In an aspect, the disclosure provides novel lipid compounds which are capable of combining with other lipid components such as neutral lipids, charged lipids, steroids and/or polymer conjugated-lipids to form lipid nanoparticles with therapeutic agents, such as oligonucleotides. Without wishing to be bound by theory, it is thought that these lipid nanoparticles shield the therapeutic agent from degradation in the serum and provide for effective delivery of the therapeutic agent to cells in vitro and in vivo.

In one embodiment, the compounds have the following structure (I):



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

G^1 and G^2 are each independently C_1 - C_6 alkylene;

L^1 and L^2 are each independently $-(C=O)-$ or $-(C=O)O-$;

R^{1a} and R^{1b} are, at each occurrence, independently either: (a) H or C_1 - C_{12} alkyl; or (b) R^{1a} is H or C_1 - C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either: (a) H or C_1 - C_{12} alkyl; or (b) R^{2a} is H or C_1 - C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{1a} and R^{ab} are, at each occurrence, independently either (a): H or C_1 - C_{12} alkyl; or (b) R^{3a} is H or C_1 - C_{12} alkyl, and R^{ab} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{4a} and R^{4b} are, at each occurrence, independently either: (a) H or C_1 - C_{12} alkyl; or (b) R^{4a} is H or C_1 - C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently H or methyl;

R^7 is $-O(C=O)R^{10}$, $-(C=O)OR^{10}$, $-NR^9(C=O)R^{10}$ or $-(C=O)NR^9R^{10}$;

R^8 is OH, $-N(R^{11})(C=O)R^{12}$, $-(C=O)NR^{11}R^{12}$, $-NR^{11}R^{12}$, $-(C=O)OR^{12}$ or $-O(C=O)R^{12}$;

R^9 is H or C_1 - C_{15} alkyl;

R^{10} is C_1 - C_{15} alkyl;

R^{11} is H or C_1 - C_6 alkyl;

R^{12} is C_1 - C_6 alkyl;

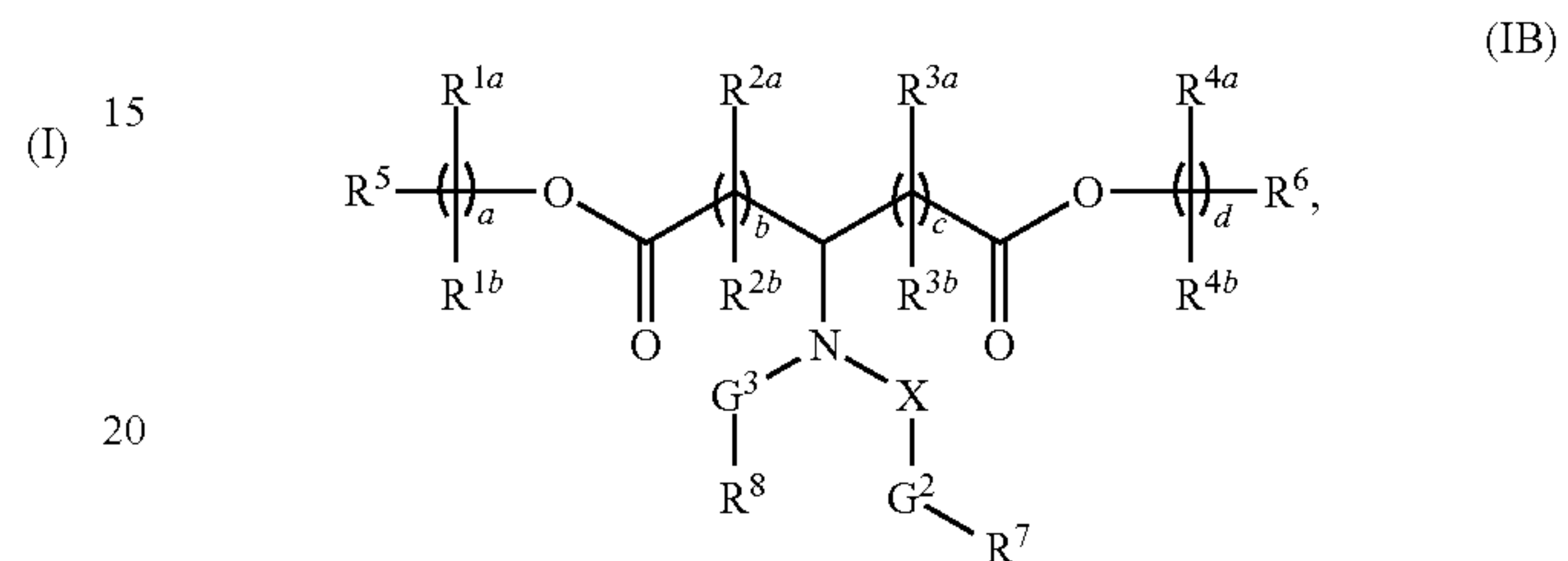
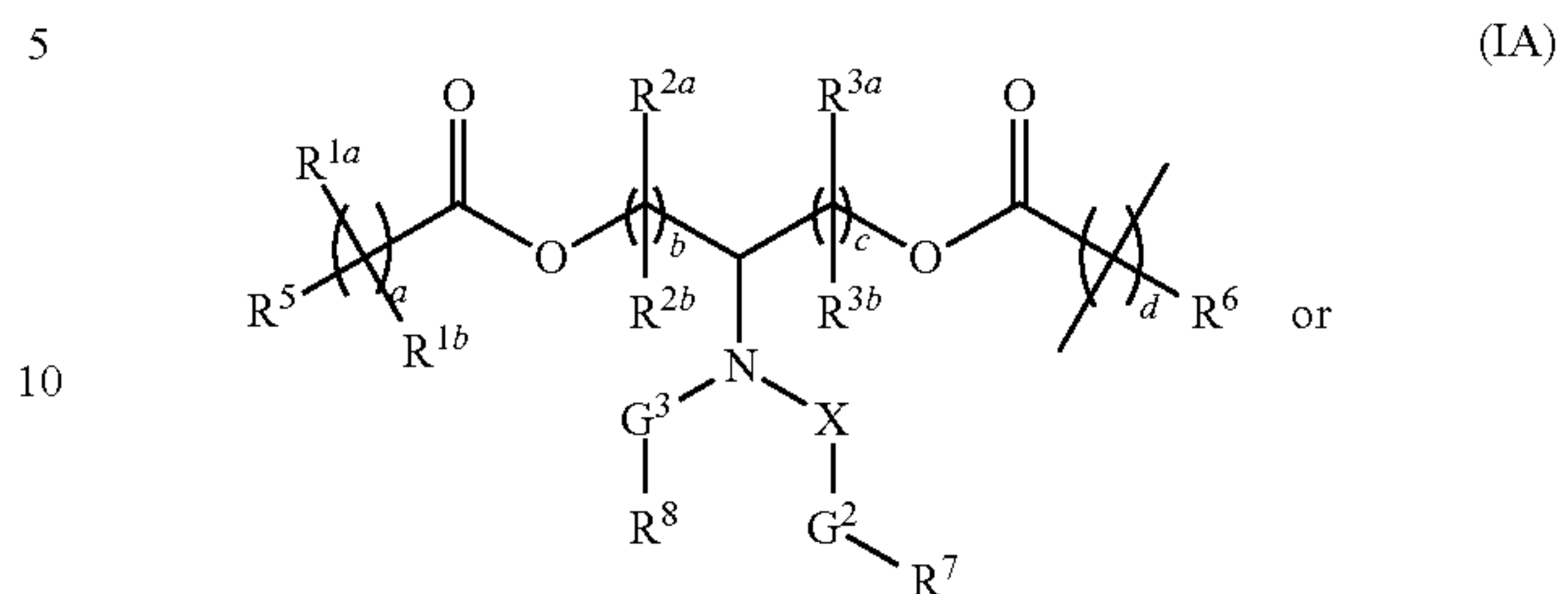
X is $-(C=O)-$ or a direct bond; and

a, b, c and d are each independently an integer from 1 to 24;

wherein each alkyl and alkylene and is independently optionally substituted.

18

In other embodiments, the compound has one of the following structures (IA) or (IB):



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof.

In certain embodiments, G^1 is C_2 - C_3 alkylene. In different embodiments, G^1 is C_4 - C_6 alkylene. For example, in various embodiments, G^1 is C_2 , C_3 , C_4 , C_5 or C_6 alkylene.

In other embodiments, G^2 is C_2 - C_4 alkylene, for example C_2 - C_3 alkylene or C_3 - C_4 alkylene. In some embodiments, G^2 is C_2 , C_3 or C_4 alkylene.

In various different embodiments, X is $-(C=O)-$, while in different embodiments X is a direct bond.

In any of the foregoing embodiments, R^7 is $-O(C=O)R^{10}$ or $-(C=O)OR^{10}$. In certain of these embodiments, R^{10} is linear C_1 - C_{15} alkyl, such as linear C_6 - C_{10} alkyl. In other such embodiments, R^{10} is methyl or R^{10} is branched C_2 - C_{15} alkyl, such as branched C_{10} - C_{15} alkyl.

In still other of the foregoing embodiments, R^7 is $-NR^9(C=O)$ or $-(C=O)NR^9R^{10}$. In some of these embodiments, R^9 is H. In other of these embodiments, R^9 and R^{10} are each independently C_6 - C_{10} alkyl.

In other embodiments, at least one occurrence of R^{1a} and R^{1b} , R^{1a} is H or C_1 - C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In more embodiments, for at least one occurrence of R^{4a} and R^{4b} , R^{4a} is H or C_1 - C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In still other embodiments, for at least one occurrence of R^{2a} and R^{2b} , R^{2a} is H or C_1 - C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

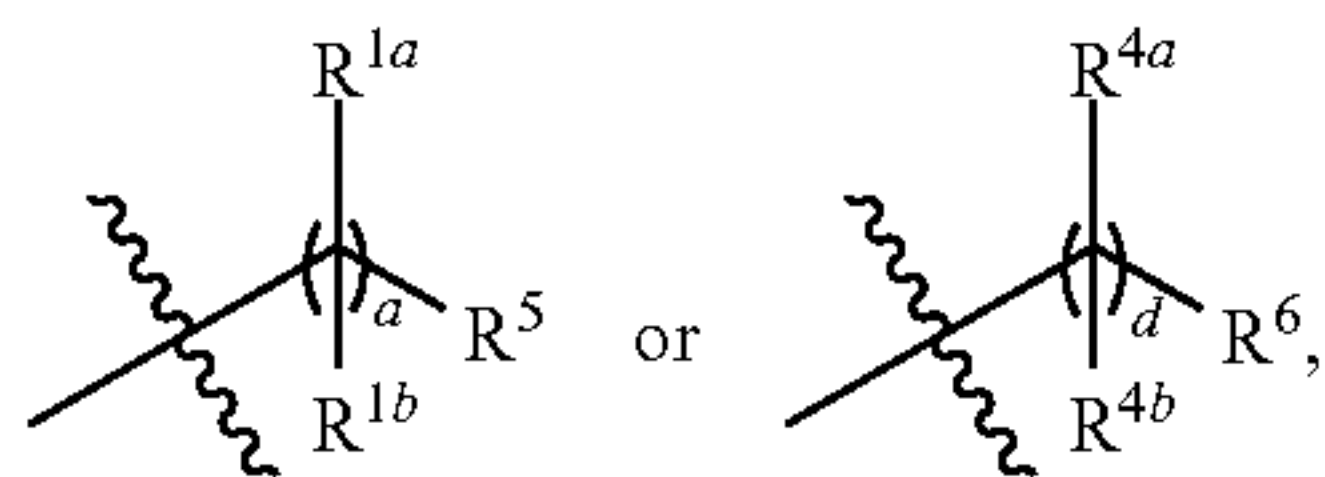
In other embodiments, for at least one occurrence of R^{1a} and R^{3b} , R^{3a} is H or C_1 - C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond.

In various of the foregoing embodiments, R^{1a} , R^{1b} , R^{2a} , R^{2b} , R^{3a} , R^{3b} , R^{4a} and R^{4b} are, at each occurrence, independently H or C_1 - C_{12} alkyl. In other embodiments, R^{2a} , R^{2b} , R^{3a} and R^{3b} are, at each occurrence, H. For example, in

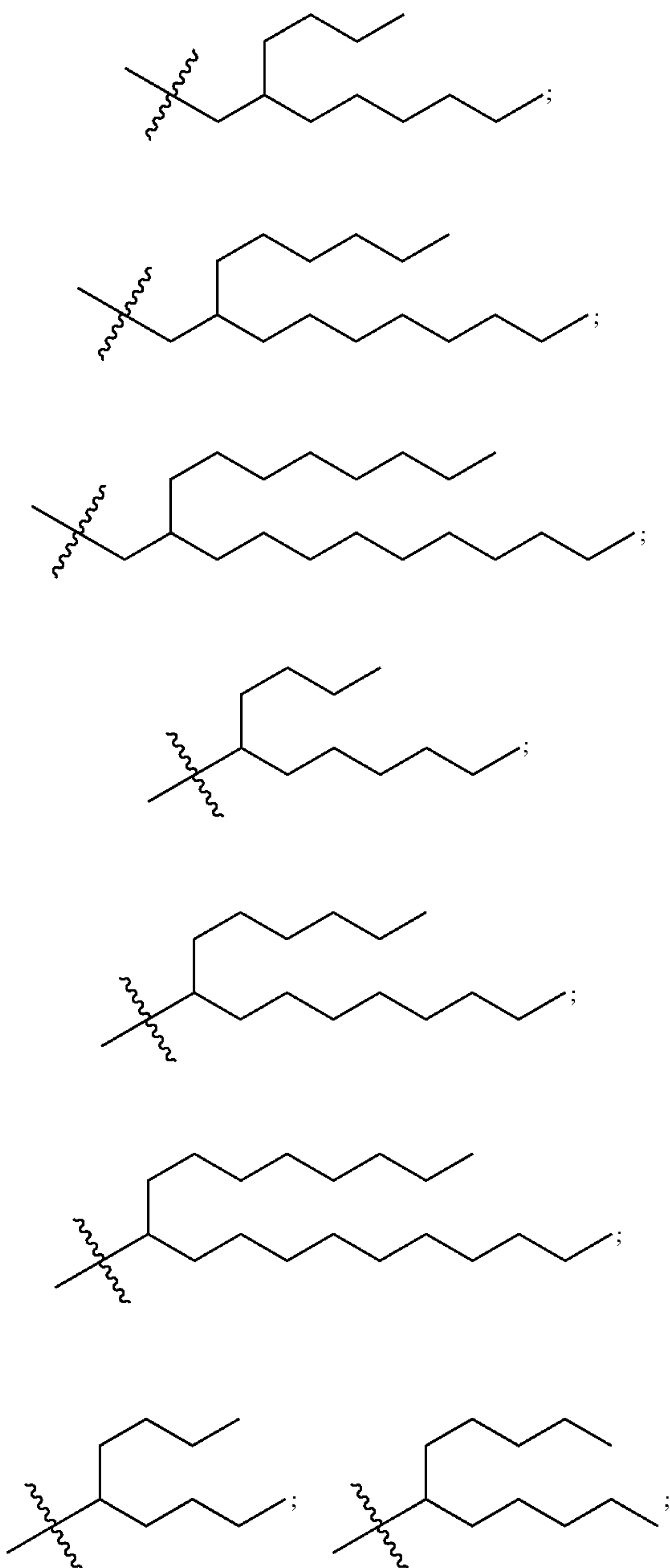
19

certain embodiment R^{1a} and R^{4a} are, at each occurrence, H. In different embodiments, at least one of R^{1b} and R^{4b} is C_1 - C_8 alkyl. For example, in some embodiments C_1 - C_8 alkyl is methyl, ethyl, n-propyl, iso-propyl, n-butyl, iso-butyl, tert-butyl, n-hexyl or n-octyl.

In other more specific embodiments,

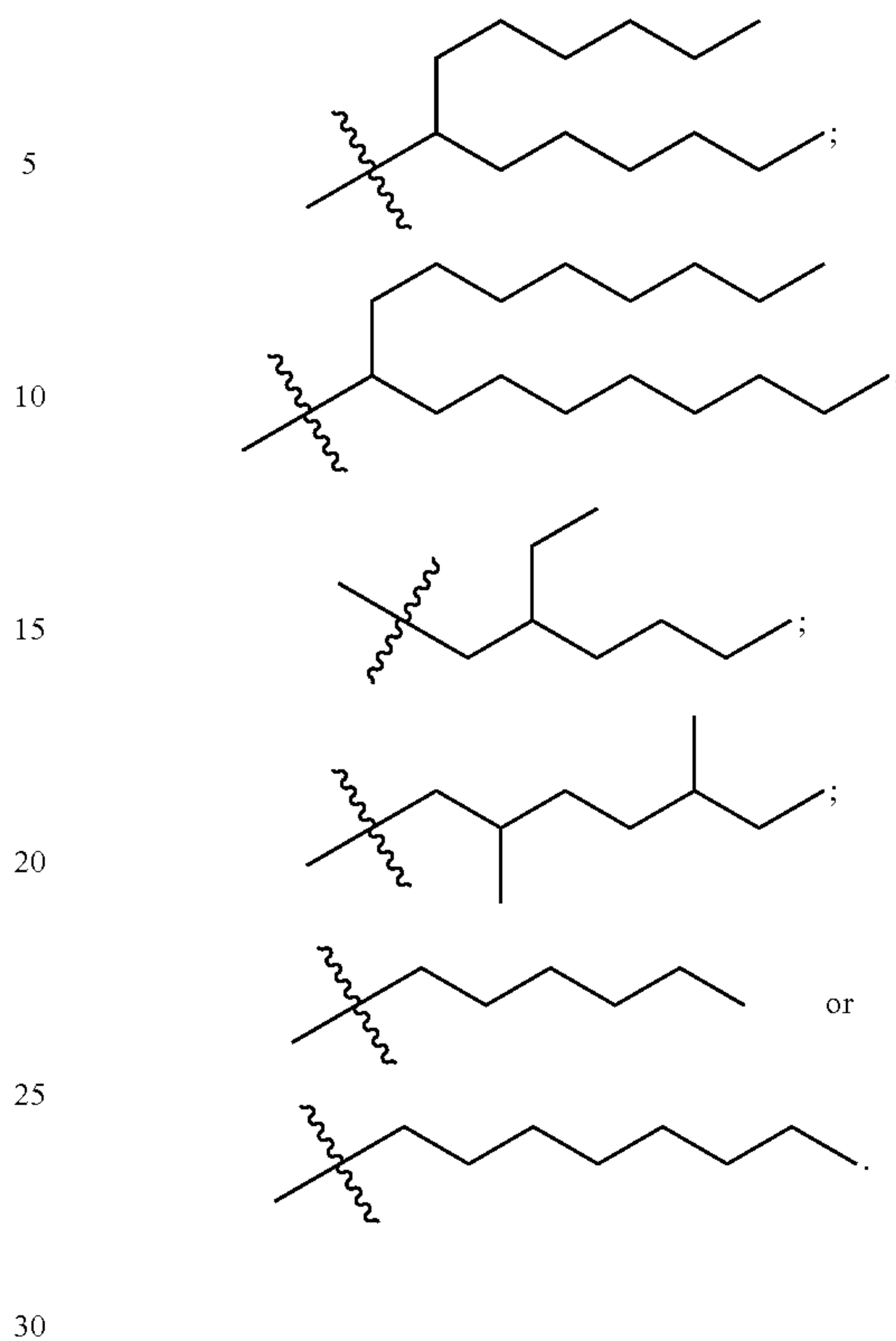


or both, independently has one of the following structures:



20

-continued



In more embodiments, a, b, c and d are each independently an integer from 2 to 12. In more embodiments, a, b, c and d are each independently an integer from 4 to 10, 5 to 10, 6 to 10, 4 to 9, 5 to 9 or 6 to 9. In still different embodiments, b and c are independently 5, 6, 7, 8, 9 or 10.

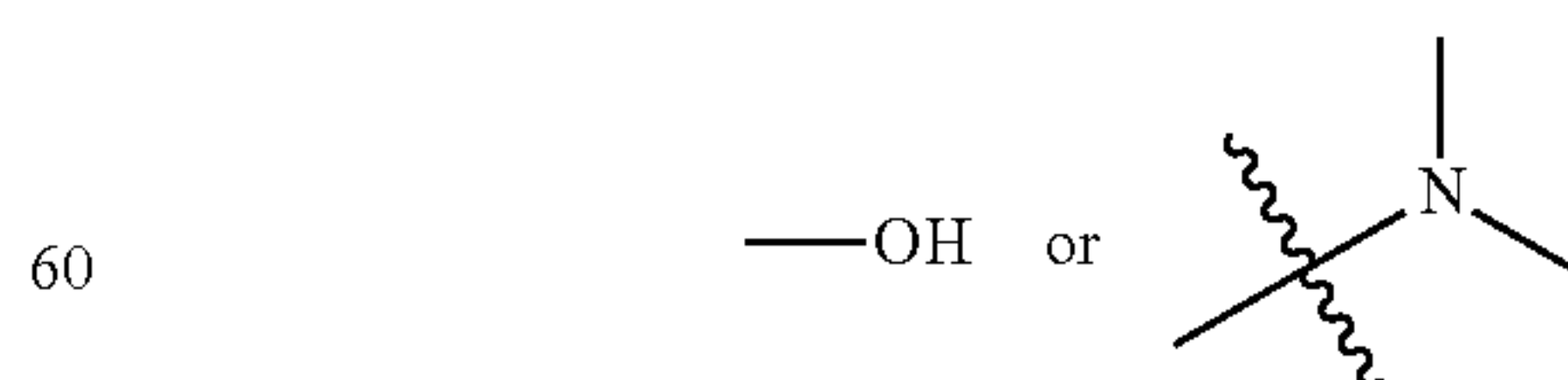
In some embodiments, one of R^5 or R^6 is methyl. In other embodiments, each of R^5 and R^6 is methyl.

In some embodiments, R^8 is OH.

In other embodiments, R^8 is $-N(R^{11})(C=O)R^{12}$. In different embodiments, R^8 is $-(C=O)NR^{11}R^{12}$. In more different embodiments, R^8 is $NR^{11}R^{12}$. In some of these foregoing embodiments, R^{11} and R^{12} are each independently H or C_1 - C_8 alkyl. In other of these embodiments, R^{11} and R^{12} are each independently H or C_1 - C_3 alkyl. For example, in some embodiments the C_1 - C_8 alkyl or C_1 - C_3 alkyl is unsubstituted or substituted with hydroxyl. In other different such embodiments, R^{11} and R^{12} are each methyl.

In other embodiments of the compound of structure (I), R^8 is $-(C=O)OR^{12}$, while in different embodiments R^8 is $-O(C=O)R^{12}$.

In specific embodiments of any of the foregoing compounds, R^8 has one of the following structures:



In various different embodiments, the disclosure provides a compound having one of the structures set forth in Table 1 below, or a pharmaceutically acceptable salt or tautomer thereof.

TABLE 1

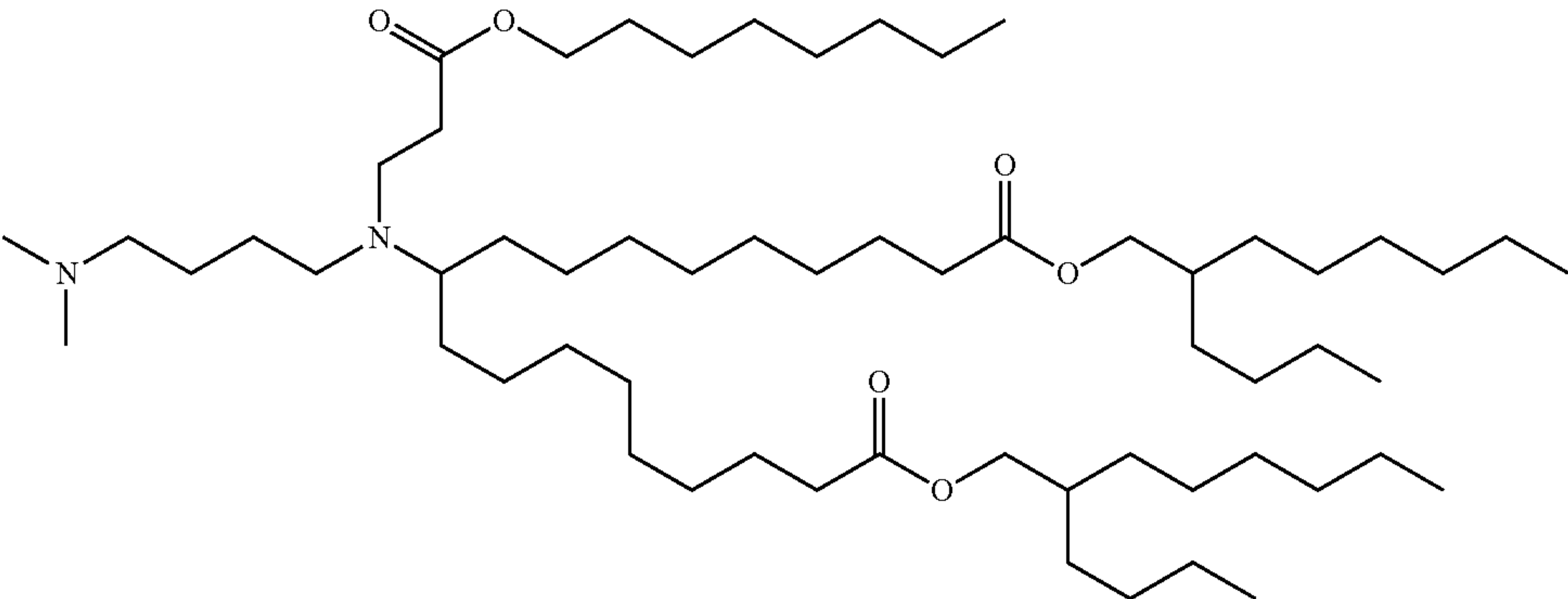
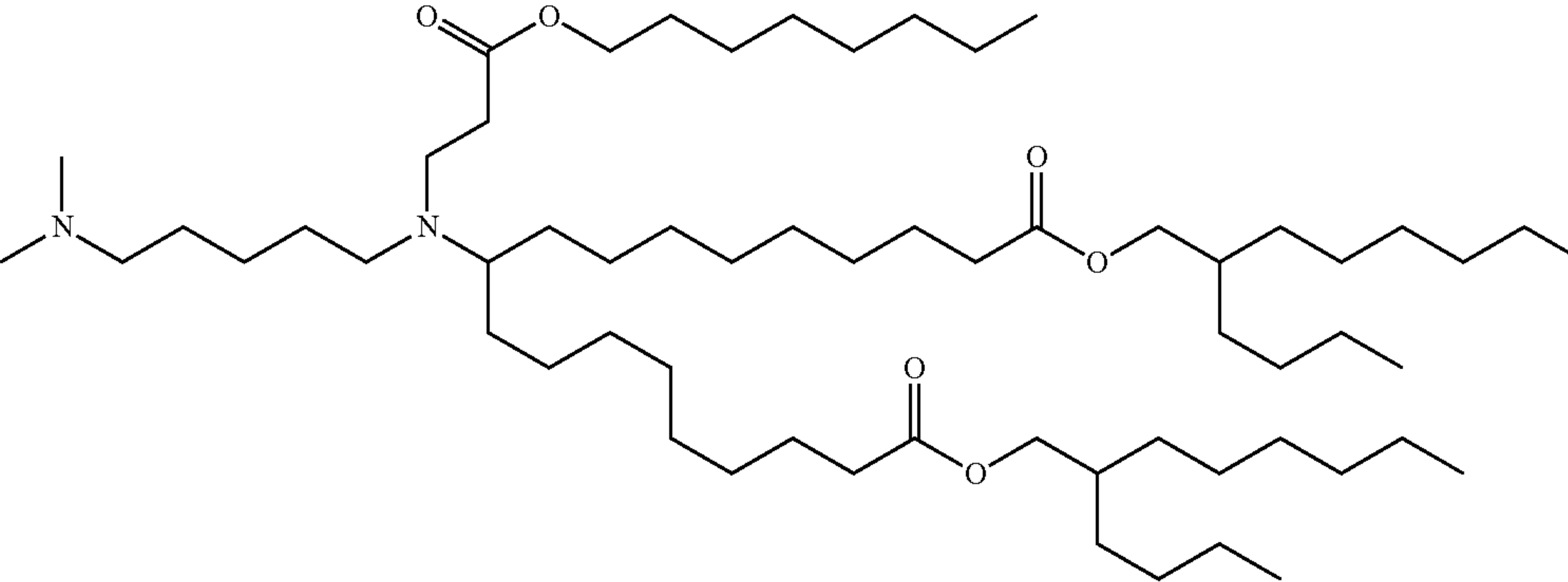
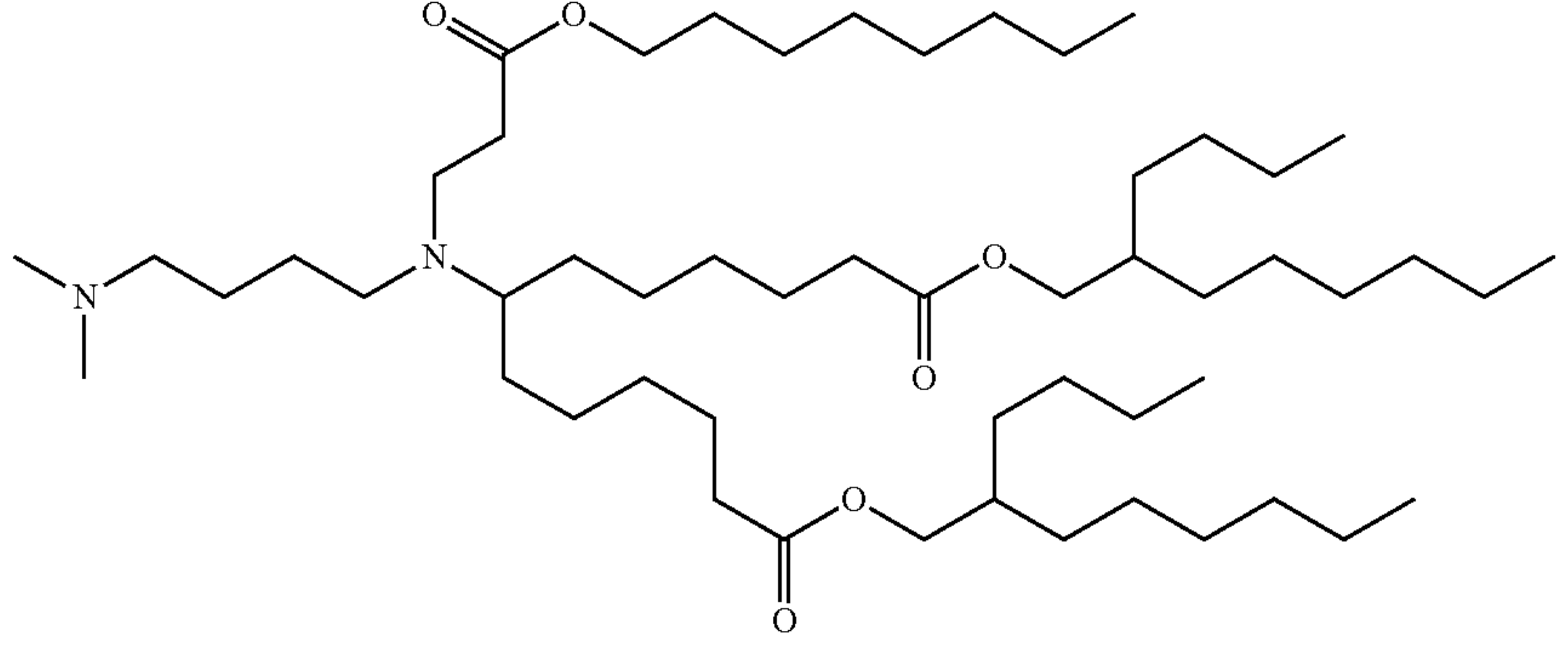
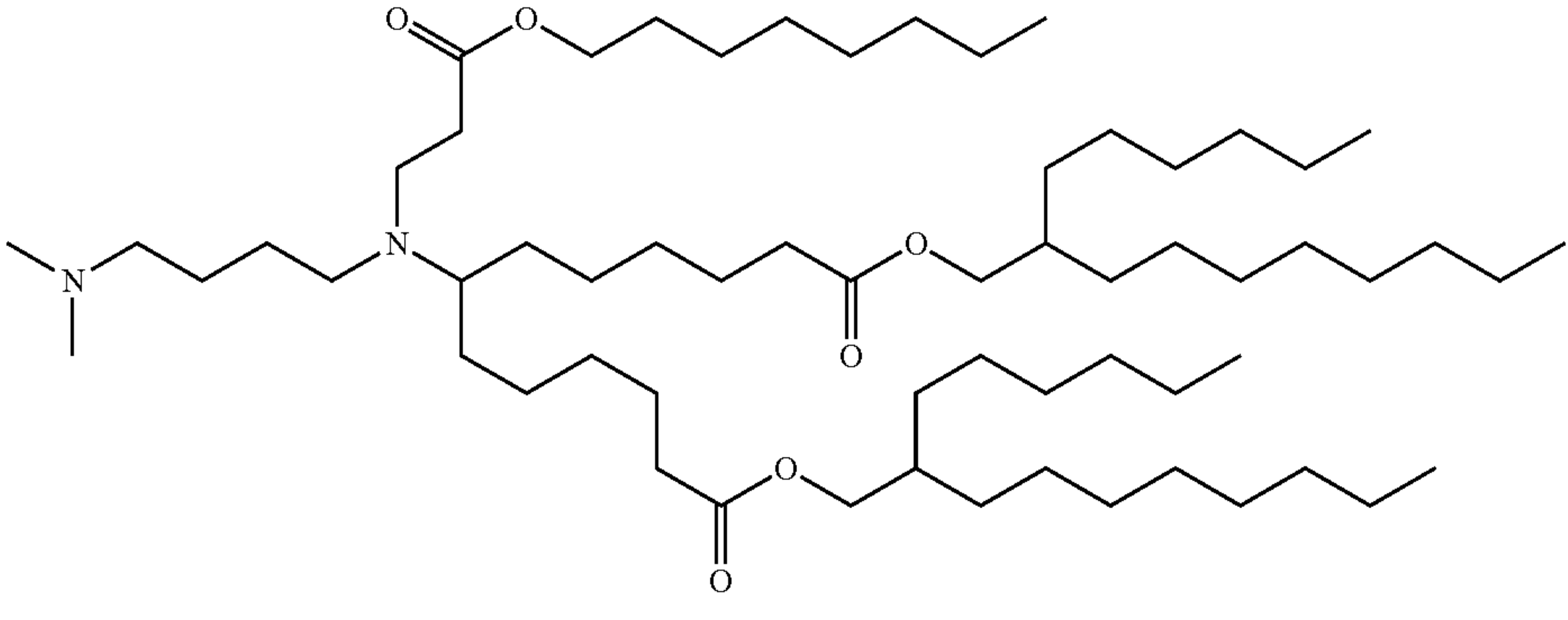
Representative Compounds	
No.	Structure
I-1	
I-2	
I-3	
I-4	

TABLE 1-continued

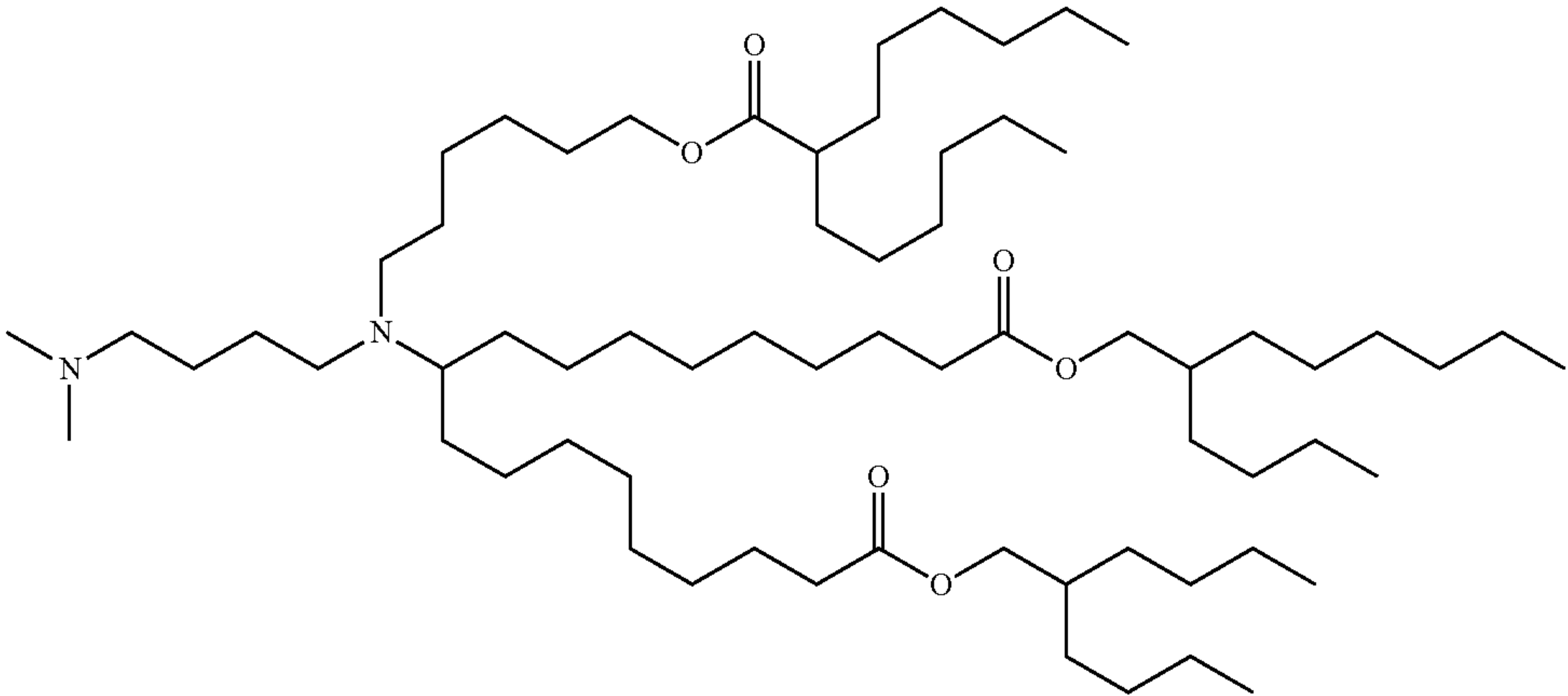
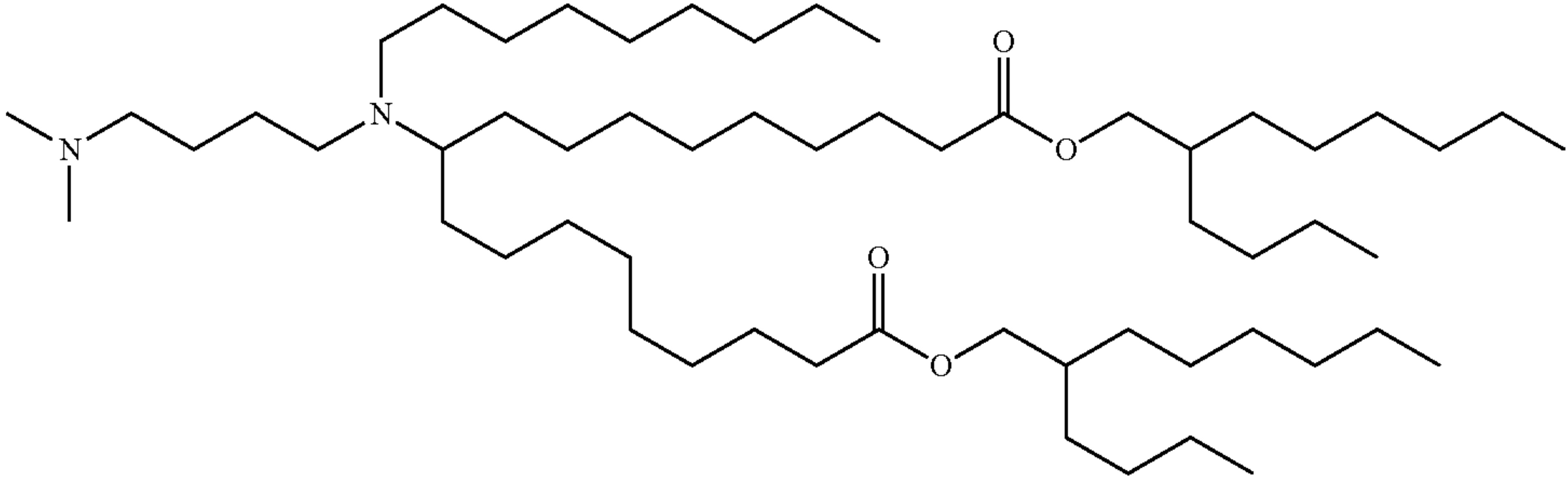
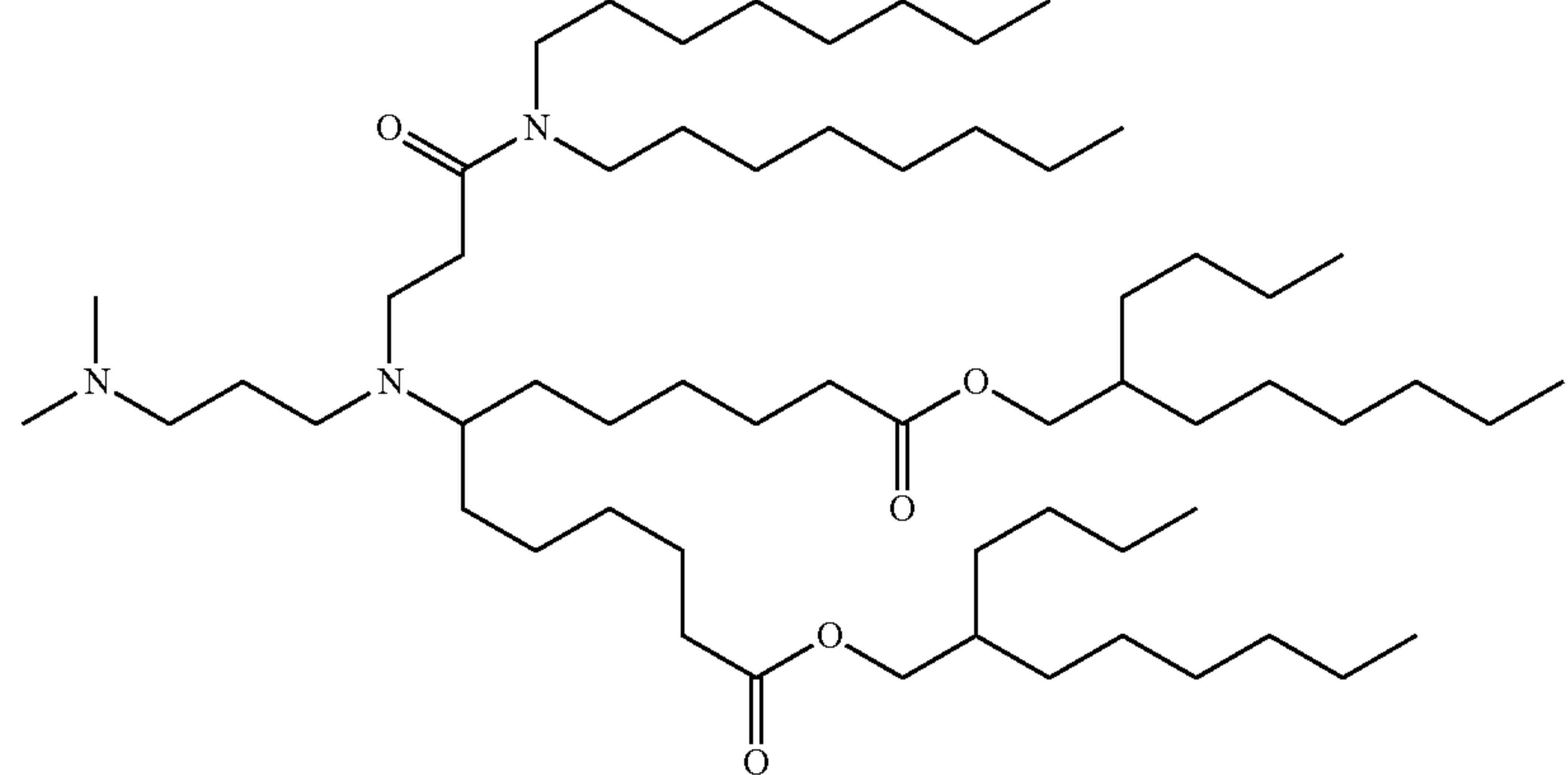
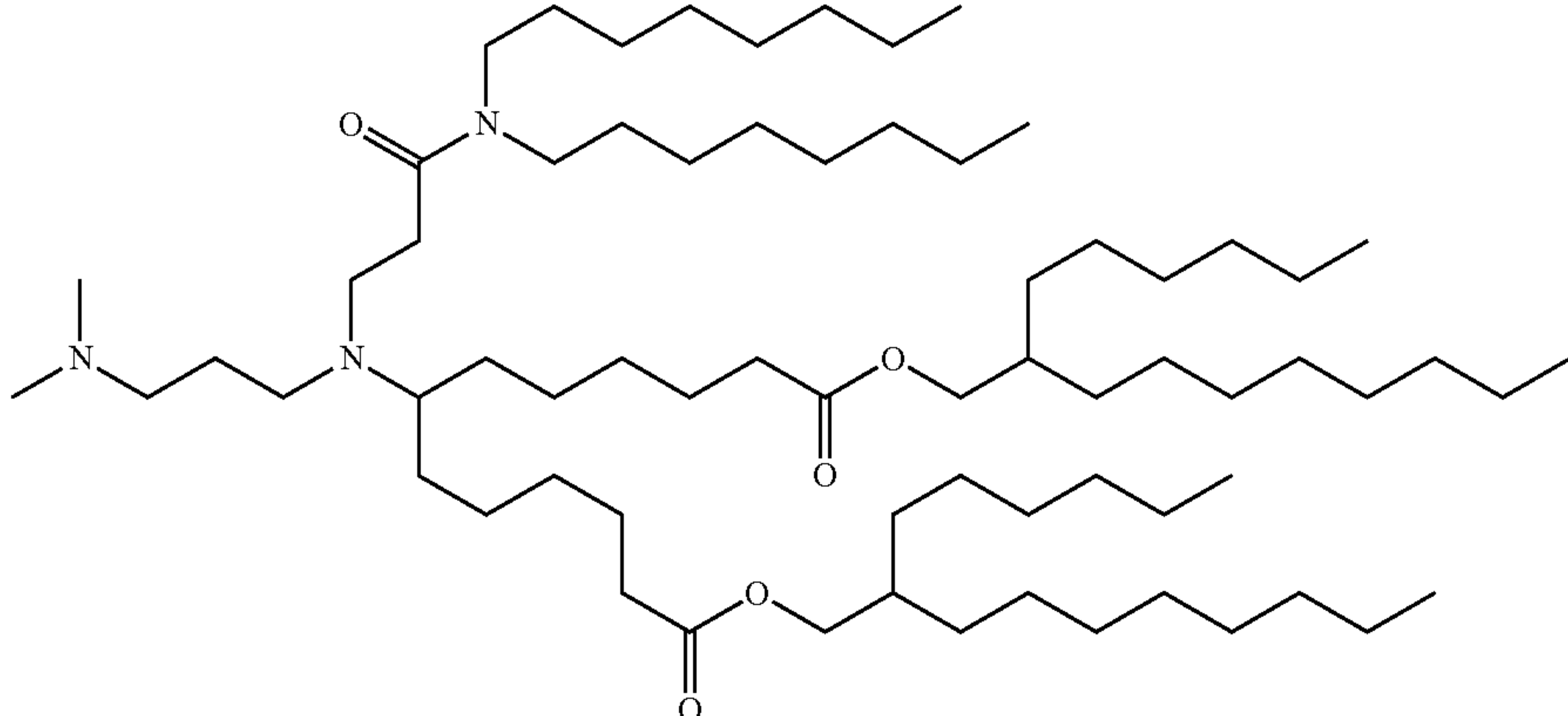
No.	Structure
I-5	
I-6	
I-7	
I-8	

TABLE 1-continued

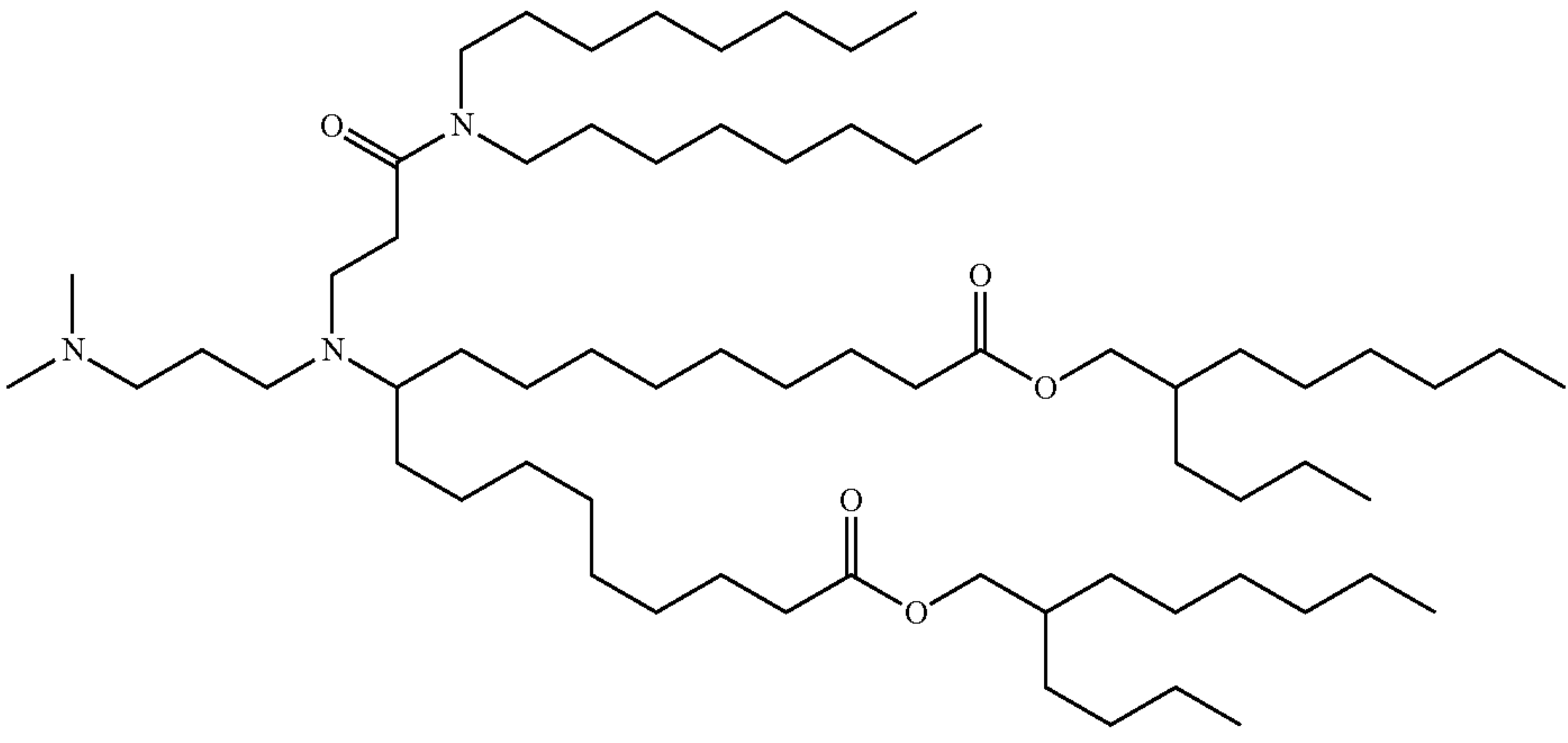
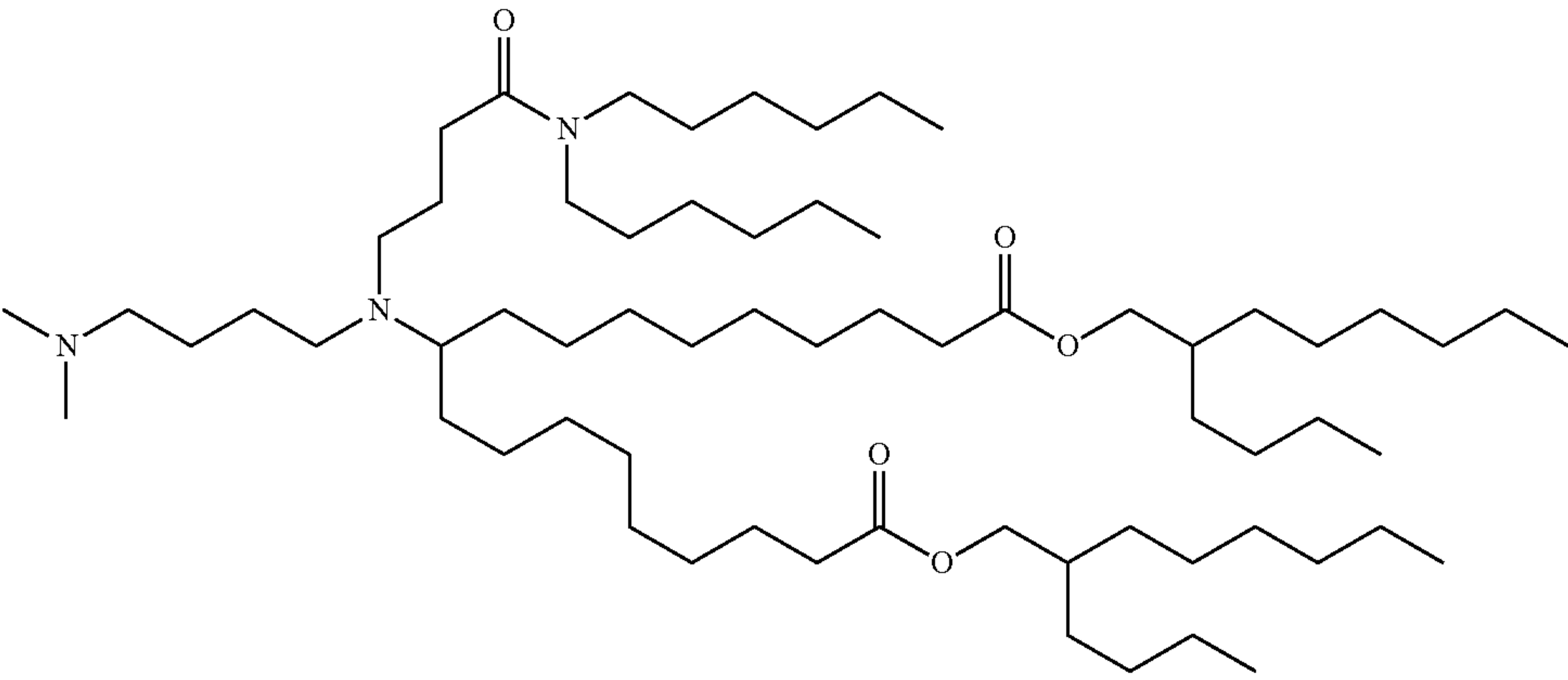
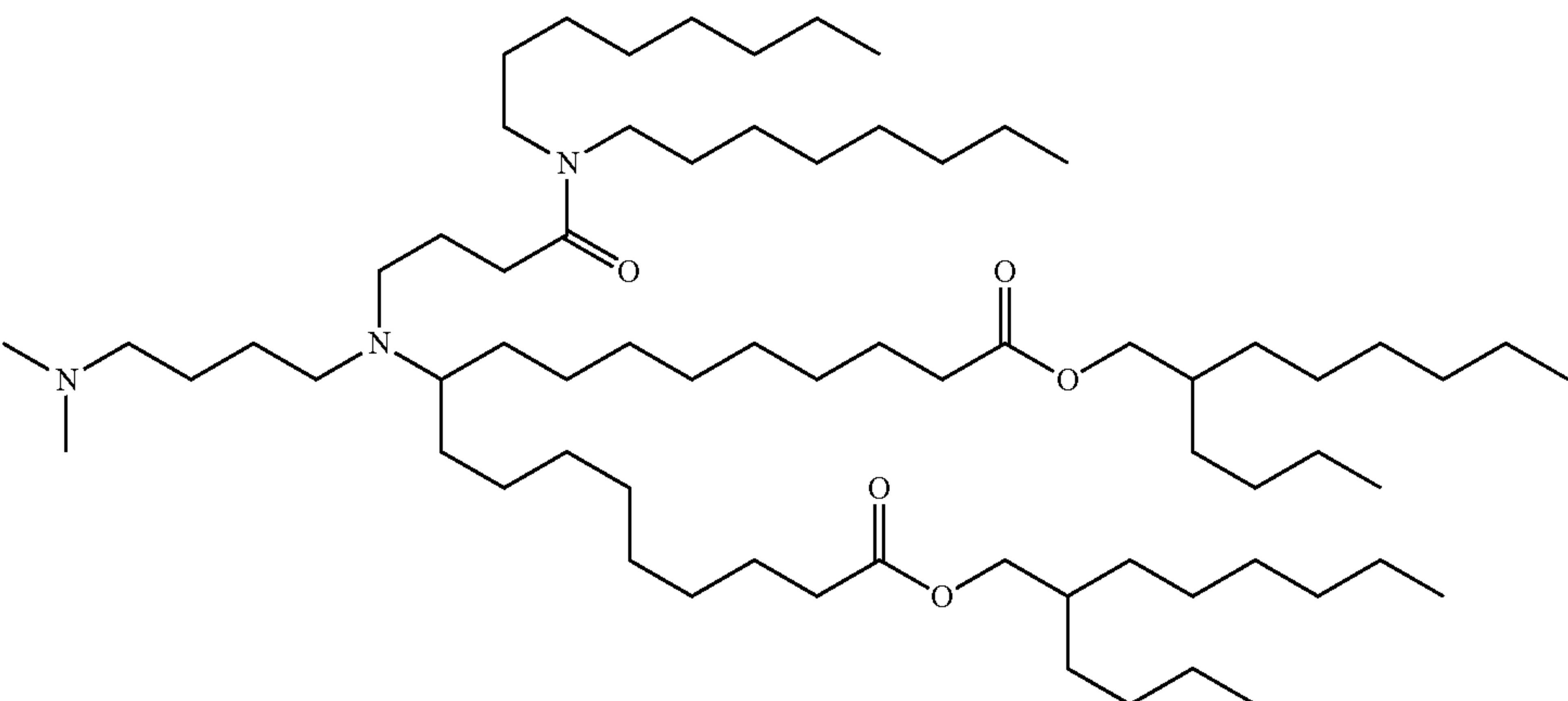
Representative Compounds	
No.	Structure
I-9	
I-10	
I-11	

TABLE 1-continued

No.	Structure
I-12	<chem>CCCCCCCCCCCC(=O)NCCN(C)CCN(C)CCCCCCCCCCCC(=O)OCCCCCCCCCCCC(=O)OCCCCCCCCCCCC</chem>
I-13	<chem>CCCCCCCCCCCC(=O)NCCCCCCCCCCCCN(C)CCN(C)CCCCCCCCCCCC(=O)OCCCCCCCCCCCC(=O)OCCCCCCCCCCCC</chem>
I-14	<chem>CCCCCCCCCCCC(=O)OC(C)CCN(C)CCN(C)CCCCCCCCCCCC(=O)OCCCCCCCCCCCC(=O)OC(C)CCCCCCCCCCCC</chem>
I-15	<chem>CCCCCCCCCCCC(=O)OC(C)CCN(C)CCN(C)CCCCCCCCCCCC(=O)OCCCCCCCCCCCC(=O)OC(C)CCCCCCCCCCCC</chem>

TABLE 1-continued

No.	Structure
I-16	
I-17	
I-18	
I-19	

TABLE 1-continued

No.	Structure
I-20	
I-21	
I-22	
I-23	

33

It is understood that any embodiment of the compounds of structure (I), as set forth above, and any specific substituent and/or variable in the compound of structure (I), as set forth above, may be independently combined with other embodiments and/or substituents and/or variables of compounds of structure (I) to form embodiments of the disclosures not specifically set forth above. In addition, in the event that a list of substituents and/or variables is listed for any particular R group, G group, L group or variable a, b, c, d or n, in a particular embodiment and/or claim, it is understood that each individual substituent and/or variable may be deleted from the particular embodiment and/or claim and that the remaining list of substituents and/or variables will be considered to be within the scope of the disclosure.

It is understood that in the present description, combinations of substituents and/or variables of the depicted formulae are permissible only if such contributions result in stable compounds.

In some embodiments, lipid nanoparticles comprising a compound of structure (I) are provided. The lipid nanoparticles optionally include excipients selected from a neutral lipid, a steroid and a polymer conjugated lipid.

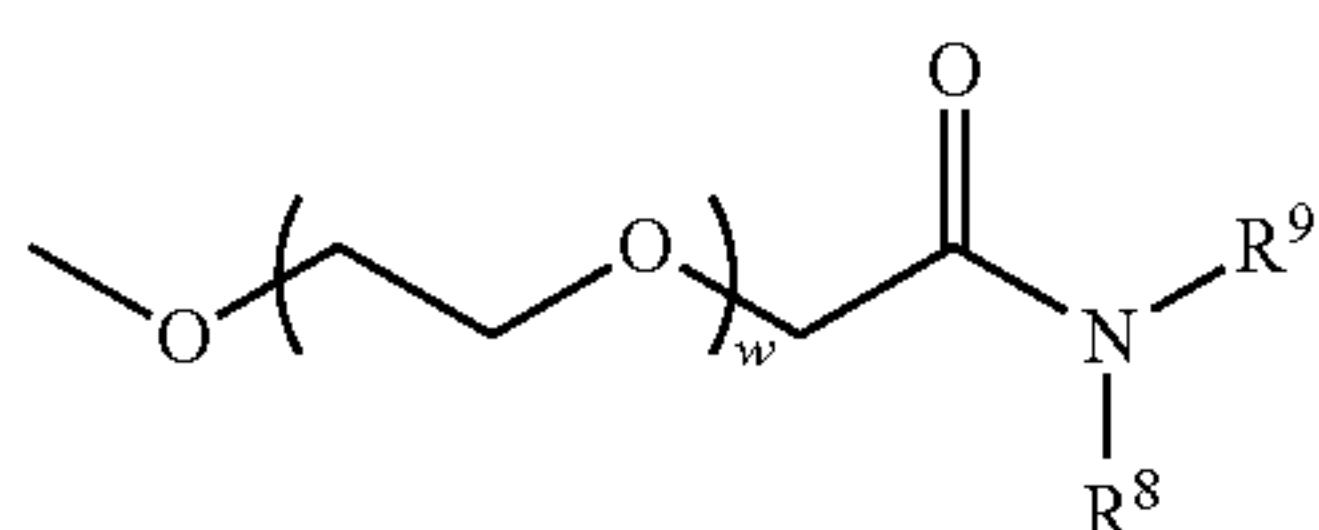
In some embodiments, compositions comprising any one or more of the compounds of structure (I) and a therapeutic agent are provided. For example, in some embodiments, the compositions comprise any of the compounds of structure (I) and a therapeutic agent and one or more excipient selected from neutral lipids, steroids and polymer conjugated lipids. Other pharmaceutically acceptable excipients and/or carriers are also included in various embodiments of the compositions.

In some embodiments, the neutral lipid is selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In some embodiments, the neutral lipid is DSPC. In various embodiments, the molar ratio of the compound to the neutral lipid ranges from about 2:1 to about 8:1.

In various embodiments, the compositions further comprise a steroid or steroid analogue. In certain embodiments, the steroid or steroid analogue is cholesterol. In some of these embodiments, the molar ratio of the compound to cholesterol ranges from about 5:1 to 1:1.

In various embodiments, the polymer conjugated lipid is a pegylated lipid. For example, some embodiments include a pegylated diacylglycerol (PEG-DAG) such as 1-(monomethoxy-polyethyleneglycol)-2,3-dimyristoylglycerol (PEG-DMG), a pegylated phosphatidylethanolamine (PEG-PE), a PEG succinate diacylglycerol (PEG-S-DAG) such as 4-O-(2',3'-di(tetradecanoyloxy)propyl)-1-O-(ω -methoxy(polyethoxy)ethyl)butanedioate (PEG-S-DMG), a pegylated ceramide (PEG-cer), or a PEG dialkoxypyrilcarbamate such as ω -methoxy(polyethoxy)ethyl-N-(2,3-di(tetradecanoyloxy)propyl)carbamate or 2,3-di(tetradecanoyloxy)propyl-N-(ω -methoxy(polyethoxy)ethyl)carbamate. In various embodiments, the molar ratio of the compound to the pegylated lipid ranges from about 100:1 to about 20:1.

In some embodiments, the composition comprises a pegylated lipid having the following structure (II):



34

or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R^8 and R^9 are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and

w has a mean value ranging from 30 to 60.

In some embodiments, R^8 and R^9 are each independently straight, saturated alkyl chains containing from 12 to 16 carbon atoms. In other embodiments, the average w ranges from about 42 to 55, for example about 49.

In some embodiments of the foregoing composition, the therapeutic agent comprises a nucleic acid. For example, in some embodiments, the nucleic acid is selected from antisense and messenger RNA. In some of the foregoing embodiments, the composition comprises a lipid nanoparticle.

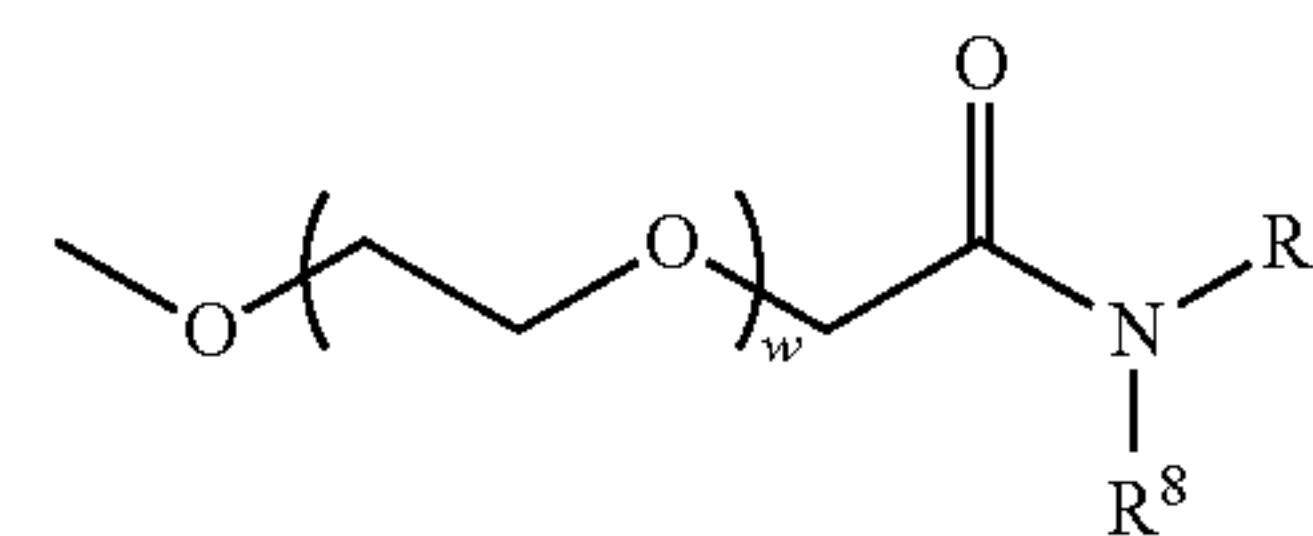
Some related embodiments provide a lipid nanoparticle comprising the compound of any one of the foregoing embodiments (e.g., a compound of structure (I)). In certain embodiments, the lipid nanoparticle further comprises a therapeutic agent (e.g., a nucleic acid such as antisense and messenger RNA).

In some embodiments, the lipid nanoparticle further comprises one or more excipient selected from neutral lipids, steroids and polymer conjugated lipids. In some embodiments, the neutral lipids are selected from DSPC, DPPC, DMPC, DOPC, POPC, DOPE and SM. In more specific embodiments, the neutral lipid is DSPC.

In some more specific embodiments, the molar ratio of the compound to the neutral lipid ranges from about 2:1 to about 8:1. In some embodiments, the steroid is cholesterol. In some embodiments, the molar ratio of the compound to cholesterol ranges from 5:1 to 1:1.

In certain embodiments, the polymer conjugated lipid is pegylated lipid. In certain more specific embodiments, the molar ratio of the compound to pegylated lipid ranges from about 100:1 to about 20:1.

In some embodiments, the pegylated lipid is PEG-DAG, PEG-PE, PEG-S-DAG, PEG-cer or a PEG dialkoxypyrilcarbamate. In other embodiments, the pegylated lipid has the following structure (II):



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

R^8 and R^9 are each independently a straight or branched, saturated or unsaturated alkyl chain containing from 10 to 30 carbon atoms, wherein the alkyl chain is optionally interrupted by one or more ester bonds; and

w has a mean value ranging from 30 to 60.

In some more specific embodiments of structure (II), R^8 and R^9 are each independently straight, saturated alkyl chains containing from 12 to 16 carbon atoms. In more specific embodiments, the average w is about 49.

In other different embodiments, the disclosure is directed to a method for administering a therapeutic agent to a patient in need thereof, the method comprising preparing or providing any of the foregoing compositions and administering the composition to the patient

For the purposes of administration, embodiments of the compounds of the present disclosure (typically in the form of lipid nanoparticles in combination with a therapeutic agent) may be administered as a raw chemical or may be formulated as pharmaceutical compositions. Pharmaceutical compositions of embodiments of the present disclosure comprise a compound of structure (I) and one or more pharmaceutically acceptable carrier, diluent or excipient. In some embodiments, the compound of structure (I) is present in the composition in an amount which is effective to form a lipid nanoparticle and deliver the therapeutic agent, e.g., for treating a particular disease or condition of interest. Appropriate concentrations and dosages can be readily determined by one skilled in the art.

Administration of the compositions of embodiments of the disclosure can be carried out via any of the accepted modes of administration of agents for serving similar utilities. The pharmaceutical compositions of embodiments of the disclosure may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suspensions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Typical routes of administering such pharmaceutical compositions include, without limitation, oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intradermal, intrasternal injection or infusion techniques. Pharmaceutical compositions of embodiments of the disclosure are formulated so as to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient in some embodiments take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of an embodiment of the disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington: The Science and Practice of Pharmacy*, 20th Edition (Philadelphia College of Pharmacy and Science, 2000). In some embodiments, the composition to be administered will, in any event, contain a therapeutically effective amount of a compound of the disclosure, or a pharmaceutically acceptable salt thereof, for treatment of a disease or condition of interest in accordance with the teachings of this disclosure.

A pharmaceutical composition of embodiments of the disclosure may be in the form of a solid or liquid. In one aspect, the carrier(s) are particulate, so that the compositions are, for example, in tablet or powder form. The carrier(s) may be liquid, with the compositions being, for example, oral syrup, injectable liquid or an aerosol, which is useful in, for example, inhalatory administration.

When intended for oral administration, the pharmaceutical composition of certain embodiments is preferably in either solid or liquid form, where semi-solid, semi-liquid, suspension and gel forms are included within the forms considered herein as either solid or liquid.

As a solid composition for oral administration, the pharmaceutical composition of some embodiments may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Such a solid composition will typically contain one or more inert diluents or edible carriers. In addition, one or more of the following may be present: binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, gum tragacanth

or gelatin; excipients such as starch, lactose or dextrans, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; and a coloring agent.

When the pharmaceutical composition of some embodiments is in the form of a capsule, for example, a gelatin capsule, it may contain, in addition to materials of the above type, a liquid carrier such as polyethylene glycol or oil.

The pharmaceutical composition of some embodiments may be in the form of a liquid, for example, an elixir, syrup, solution, emulsion or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred compositions contain, in addition to a compound of structure (I), one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent may be included.

The liquid pharmaceutical compositions of embodiments of the disclosure, whether they be solutions, suspensions or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose; agents to act as cryoprotectants such as sucrose or trehalose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

The pharmaceutical composition of embodiments of the disclosure may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, bee wax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device.

The pharmaceutical composition of embodiments of the disclosure may include various materials, which modify the physical form of a solid or liquid dosage unit. For example, the composition may include materials that form a coating shell around the active ingredients. The materials that form the coating shell are typically inert, and may be selected from, for example, sugar, shellac, and other enteric coating agents. Alternatively, the active ingredients may be encased in a gelatin capsule.

The pharmaceutical composition of embodiments of the disclosure in solid or liquid form may include an agent that binds to the compound of the disclosure and thereby assists in the delivery of the LNP. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, or a protein.

The pharmaceutical composition of embodiments of the disclosure may consist of dosage units that can be administered as an aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active ingredients. Aerosols of LNPs of embodiments of the disclosure may be delivered in single phase, bi-phasic, or tri-phasic systems in order to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, sub-containers, and the like, which together may form a kit. One skilled in the art, without undue experimentation, may determine preferred aerosols.

The pharmaceutical compositions of embodiments of the disclosure may be prepared by methodology well known in the pharmaceutical art. For example, a pharmaceutical composition intended to be administered by injection can be prepared by combining the lipid nanoparticles of the disclosure with sterile, distilled water or other carrier so as to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with the compound of the disclosure so as to facilitate dissolution or homogeneous suspension of the compound in the aqueous delivery system.

The compositions of embodiments of the disclosure, or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount, which will vary depending upon a variety of factors including the activity of the specific therapeutic agent employed; the metabolic stability and length of action of the therapeutic agent; the age, body weight, general health, sex, and diet of the patient; the mode and time of administration; the rate of excretion; the drug combination; the severity of the particular disorder or condition; and the subject undergoing therapy.

Compositions of embodiments of the disclosure may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single pharmaceutical dosage formulation of a composition of embodiments of the disclosure and one or more additional active agents, as well as administration of the composition of embodiments of the disclosure and each active agent in its own separate pharmaceutical dosage formulation. For example, a composition of embodiments of the disclosure and the other active agent can be administered to the patient together in a single oral dosage composition such as a tablet or capsule, or each agent administered in separate oral dosage formulations. Where separate dosage formulations are used, the compounds of embodiments of the disclosure and one or more additional active agents can be administered at essentially the same time, i.e., concurrently, or at separately staggered times, i.e., sequentially; combination therapy is understood to include all these regimens.

Preparation methods for the above compounds and compositions are described herein below and/or known in the art.

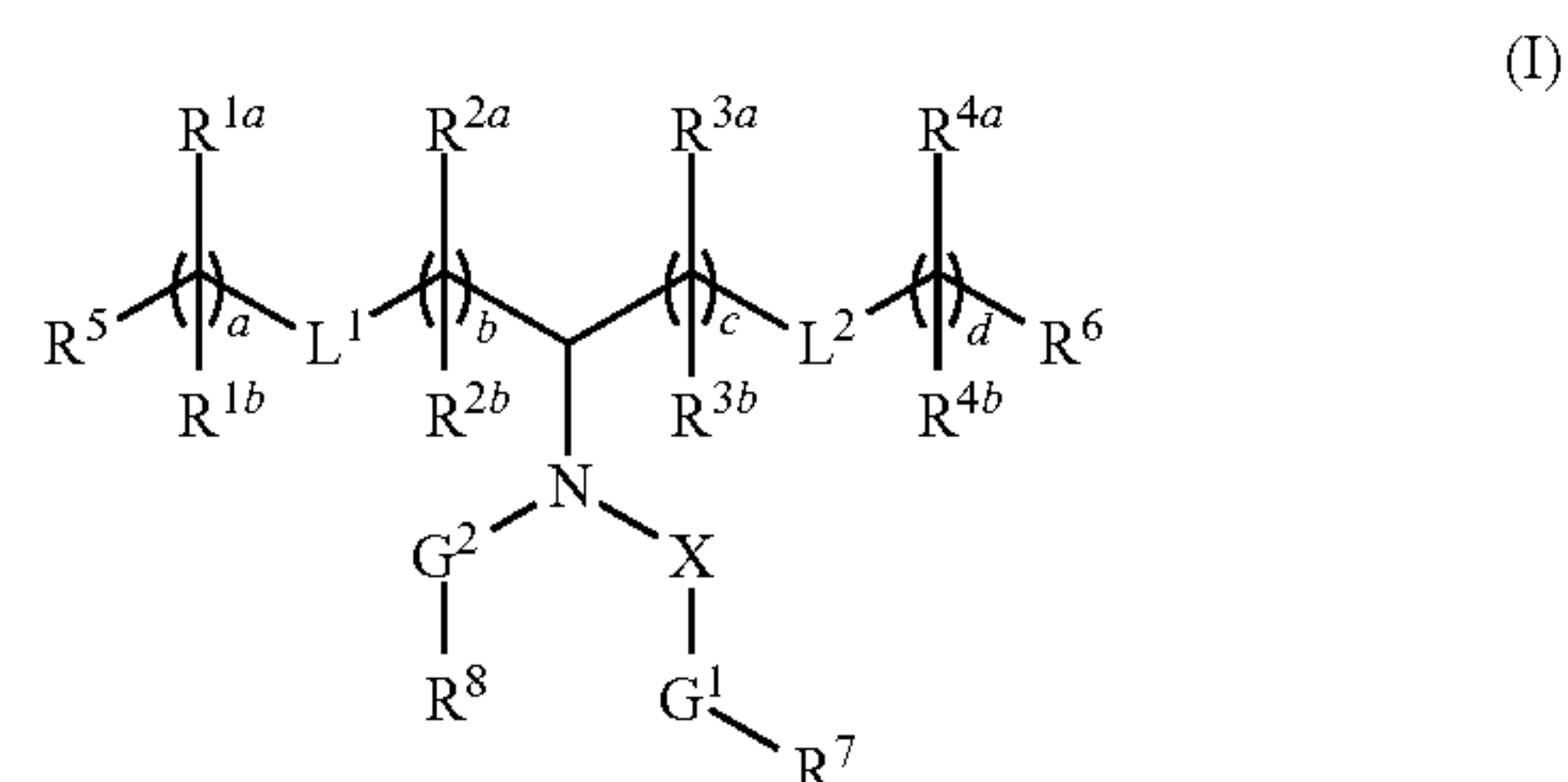
It will be appreciated by those skilled in the art that in the process described herein the functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto and carboxylic acid. Suitable protecting

groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, t-butyldimethylsilyl, t-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include t-butoxycarbonyl, benzyloxycarbonyl, and the like. Suitable protecting groups for mercapto include —C(O)—R" (where R" is alkyl, aryl or arylalkyl), p-methoxybenzyl, trityl and the like. Suitable protecting groups for carboxylic acid include alkyl, aryl or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T. W. and P. G. M. Wutz, *Protective Groups in Organic Synthesis* (1999), 3rd Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or 2-chlorotrityl-chloride resin.

It will also be appreciated by those skilled in the art, although such protected derivatives of compounds of this disclosure may not possess pharmacological activity as such, they may be administered to a mammal and thereafter metabolized in the body to form compounds of the disclosure which are pharmacologically active. Such derivatives may therefore be described as "prodrugs." All prodrugs of compounds of this disclosure are included within the scope of the disclosure.

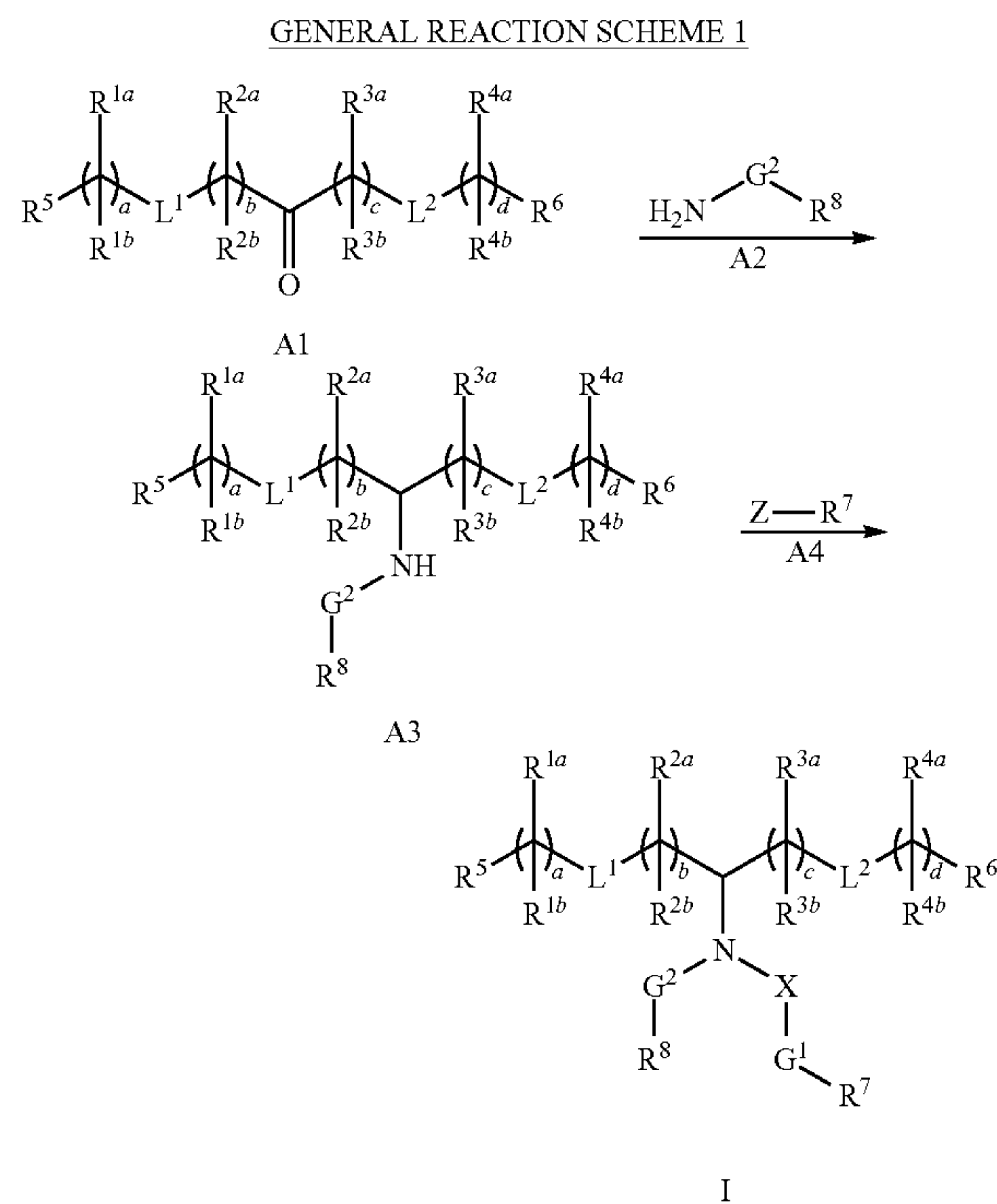
Furthermore, compounds of embodiments of the disclosure which exist in free base or acid form can be converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to one skilled in the art. Salts of compounds of embodiments of the disclosure can be converted to their free base or acid form by standard techniques.

The following General Reaction Scheme 1 illustrates exemplary methods to make compounds of this disclosure, i.e., compounds of structure (I):



or a pharmaceutically acceptable salt, tautomer, or stereoisomer thereof, wherein a, b, c, d, G¹, G², L¹, L², R^{1a}, R^{1b}, R^{2a}, R^{2b}, R^{3a}, R^{3b}, R^{4a}, R^{4b}, R⁵, R⁶, R⁷, R⁸ and X are as defined herein. It is understood that one skilled in the art may be able to make these compounds by similar methods or by combining other methods known to one skilled in the art. It is also understood that one skilled in the art would be able to make, in a similar manner as described below, other compounds of structure (I) not specifically illustrated below by using the appropriate starting components and modifying the parameters of the synthesis as needed. In general, starting components may be obtained from sources such as Sigma Aldrich, Lancaster Synthesis, Inc., Maybridge, Matrix Scientific, TCI, and Fluorochem USA, etc. or synthesized according to sources known to those skilled in the art (see, for example, *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, 5th edition (Wiley, December 2000)) or prepared as described in this disclosure.

39



General Reaction Scheme I provides an exemplary method for preparation of a compound of structure (I) (i.e., A5) a, b, c, d, G², L¹, L², R^{1a}, R^{1b}, R^{2a}, R^{2b}, R^{3a}, R^{3b}, R^{4a}, R^{4b}, R⁵, R⁶, R⁸, R⁹ and R¹⁰ in General reaction Scheme 1 are as defined herein, and Z represents an activated analogue of G¹ sufficient for bond formation with the NH group of A3 (e.g., an alkene or an alkylene terminating in an aldehyde, acid halide, acrylate, etc.). Intermediates and reagents (e.g., A1 and A2) needed for preparation of the compounds according to General Reaction Scheme I can be purchased or prepared according to the examples below or methods known by one of ordinary skill in the art.

It should be noted that various alternative strategies for preparation of compounds of structure (I) are available to those of ordinary skill in the art. For example, other compounds of structure (I) wherein can be prepared according to analogous methods using the appropriate starting material. The use of protecting groups as needed and other modification to the above General Reaction Scheme will be readily apparent to one of ordinary skill in the art.

The following examples are provided for purpose of illustration and not limitation.

Example 1

Luciferase mRNA In Vivo Evaluation Using the Lipid Nanoparticle Compositions

Lipid nanoparticles were prepared and tested according to the general procedures described in PCT Pub. Nos. WO 2015/199952 and WO 2017/004143, the full disclosures of which are incorporated herein by reference. Briefly, cationic lipid, DSPC, cholesterol and PEG-lipid were solubilized in

40

ethanol at a molar ratio of about 50:10:38.5:1.5 or about 47.5:10:40.8:1.7. Lipid nanoparticles (LNP) were prepared at a total lipid to mRNA weight ratio of approximately 10:1 to 30:1. The mRNA is diluted to 0.2 mg/mL in 10 to 50 mM citrate or acetate buffer, pH 4. Syringe pumps were used to mix the ethanolic lipid solution with the mRNA aqueous solution at a ratio of about 1:5 to 1:3 (vol/vol) with total flow rates above 15 mL/min. The ethanol was then removed and the external buffer replaced with PBS by dialysis. Finally, the lipid nanoparticles were filtered through a 0.2 μm pore sterile filter. Lipid nanoparticle particle size was approximately 55-95 nm diameter, and in some instances approximately 70-90 nm diameter as determined by quasi-elastic light scattering using a Malvern Zetasizer Nano ZS (Malvern, UK).

Studies were performed in 6-8 week old female C₅₇BL/6 mice (Charles River) or 8-10 week old CD-1 (Harlan) mice (Charles River) according to guidelines established by an institutional animal care committee (ACC) and the Canadian Council on Animal Care (CCAC). Varying doses of mRNA-lipid nanoparticle were systemically administered by tail vein injection and animals euthanized at a specific time point (e.g., 4 hours) post-administration. Liver and spleen were collected in pre-weighed tubes, weights determined, immediately snap frozen in liquid nitrogen and stored at -80° C. until processing for analysis.

For liver, approximately 50 mg was dissected for analyses in a 2 mL FastPrep tubes (MP Biomedicals, Solon Ohio). 1/4" ceramic sphere (MP Biomedicals) is added to each tube and 500 μL of Glo Lysis Buffer—GLB (Promega, Madison Wis.) equilibrated to room temperature is added to liver tissue. Liver tissues were homogenized with the FastPrep24 instrument (MP Biomedicals) at 2×6.0 m/s for 15 seconds. Homogenate was incubated at room temperature for 5 minutes prior to a 1:4 dilution in GLB and assessed using SteadyGlo Luciferase assay system (Promega). Specifically, 50 μL of diluted tissue homogenate was reacted with 50 μL of SteadyGlo substrate, shaken for 10 seconds followed by 5 minute incubation and then quantitated using a CentroXS³ LB 960 luminometer (Berthold Technologies, Germany). The amount of protein assayed was determined by using the BCA protein assay kit (Pierce, Rockford Ill.). Relative luminescence units (RLU) were then normalized to total μg protein assayed. To convert RLU to ng luciferase a standard curve was generated with QuantiLum Recombinant Luciferase (Promega).

The FLuc mRNA (L-6107 or L-7202) from Trilink Biotechnologies will express a luciferase protein, originally isolated from the firefly, *Photinus pyralis*. FLuc is commonly used in mammalian cell culture to measure both gene expression and cell viability. It emits bioluminescence in the presence of the substrate, luciferin. This capped and polyadenylated mRNA was fully substituted with respect to uridine and/or cytosine nucleosides.

Example 2

Determination of pK_a of Formulated Lipids

As described elsewhere, the pK_a of formulated cationic lipids is correlated with the effectiveness of LNPs for

41

delivery of nucleic acids (see Jayaraman et al, *Angewandte Chemie*, International Edition (2012), 51(34), 8529-8533; Semple et al, *Nature Biotechnology* 28, 172-176 (2010)). The preferred range of pK_a is ~ 5 to ~ 7 . The pK_a of each cationic lipid was determined in lipid nanoparticles using an assay based on fluorescence of 2-(p-toluidino)-6-naphthalene sulfonic acid (TNS). Lipid nanoparticles comprising cationic lipid/DSPC/cholesterol/PEG-lipid (50/10/38.5/1.5 mol %) in PBS at a concentration of 0.4 mM total lipid were prepared using the in-line process as described in Example 1. TNS was prepared as a 100 μ M stock solution in distilled water. Vesicles were diluted to 24 μ M lipid in 2 mL of buffered solutions containing, 10 mM HEPES, 10 mM IVIES, 10 mM ammonium acetate, 130 mM NaCl, where the pH ranged from 2.5 to 11. An aliquot of the TNS solution was added to give a final concentration of 1 μ M and following vortex mixing fluorescence intensity was measured at room temperature in a SLM Aminco Series 2 Luminescence Spectrophotometer using excitation and emission wavelengths of 321 nm and 445 nm. A sigmoidal best fit analysis was applied to the fluorescence data and the pK_a was measured as the pH giving rise to half-maximal fluorescence intensity.

42

Example 3

Determination of Efficacy of Lipid Nanoparticle Formulations Containing Various Cationic Lipids Using an In Vivo Luciferase mRNA Expression Rodent Model

Representative compounds of the disclosure shown in Table 2 were formulated using the following molar ratio: 50% cationic lipid/10% distearoylphosphatidylcholine (DSPC)/38.5% Cholesterol/1.5% PEG lipid 2-[2-(ω -methoxy(polyethyleneglycol₂₀₀₀)ethoxy]-N,N-ditetradecylacetamide) or 47.5% cationic lipid/10% DSPC/40.7% Cholesterol/1.8% PEG lipid. Relative activity was determined by measuring luciferase expression in the liver 4 hours following administration via tail vein injection as described in Example 1. The activity was compared at a dose of 1.0 or 0.5 mg mRNA/kg and expressed as ng luciferase/g liver measured 4 hours after administration, as described in Example 1. Compound numbers in Table 2 refer to the compound numbers of Table 1.

TABLE 2

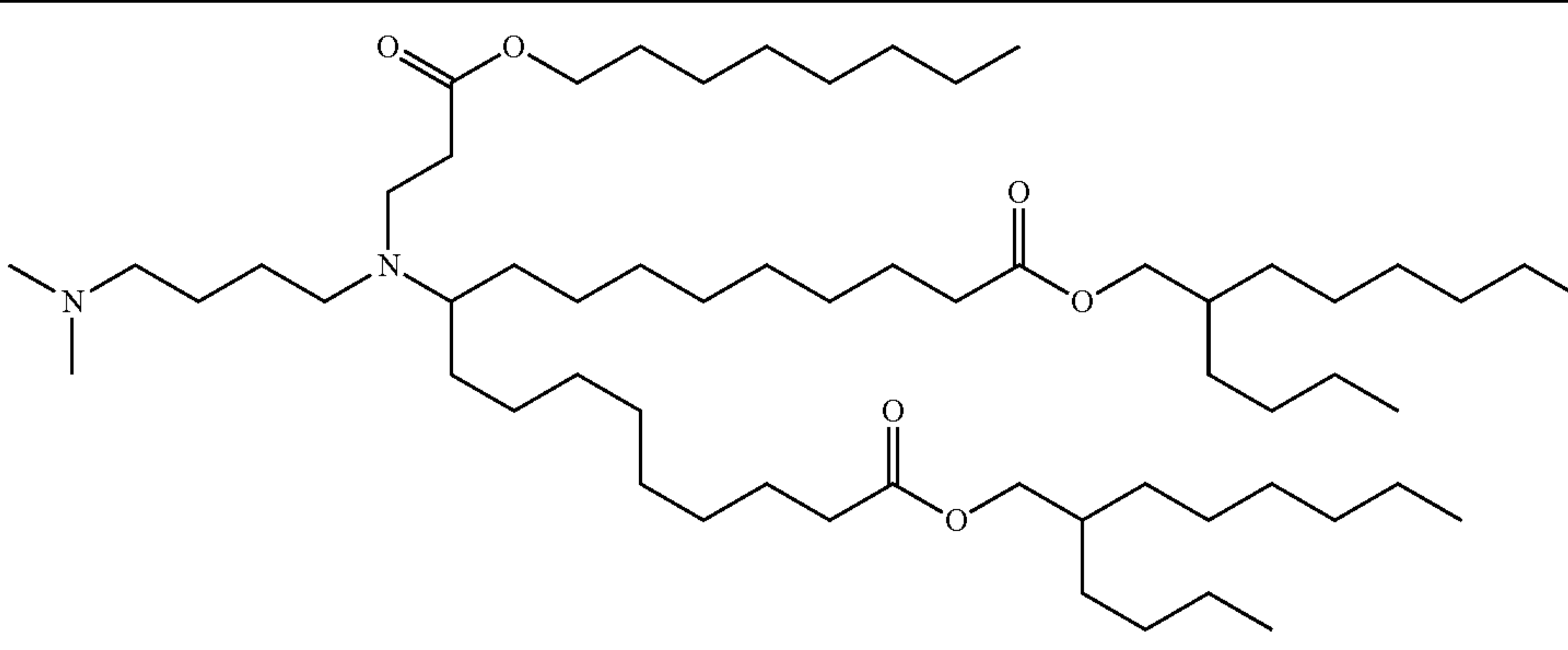
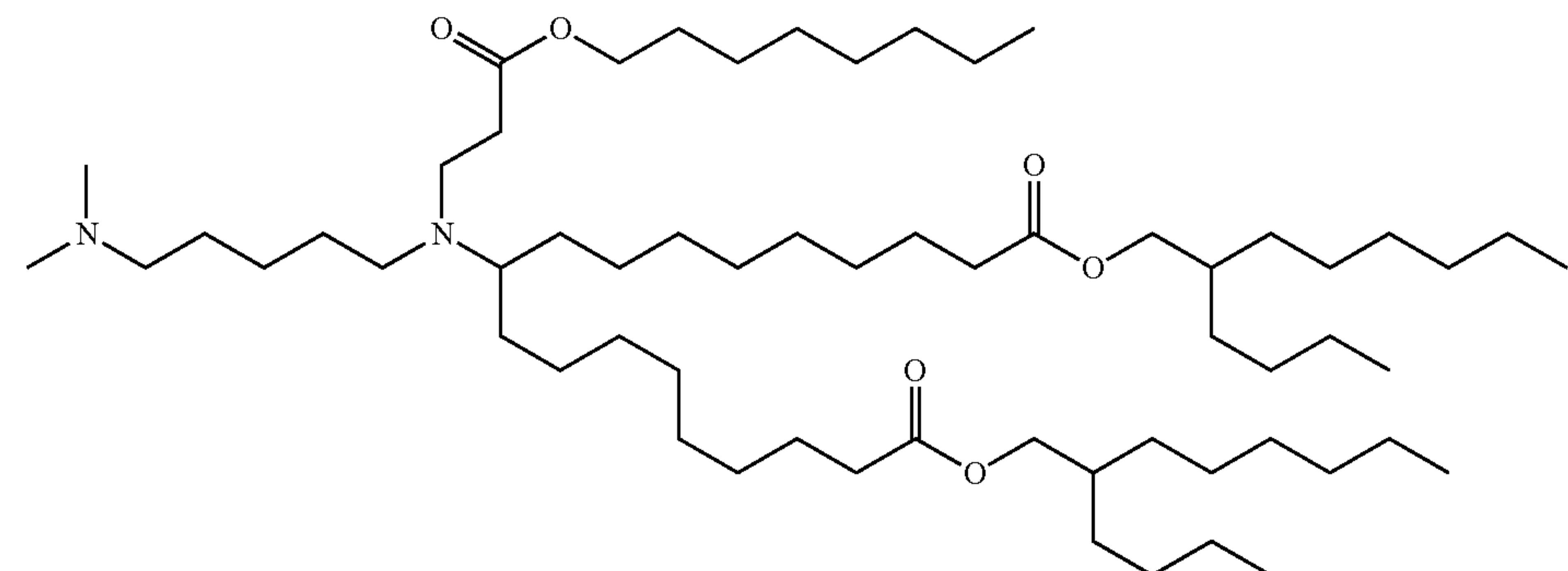
Novel Cationic Lipids and Associated Activity			
Cmp. No.	pKa	Liver Luc @ 1.0 mg/kg (ng luc/g liver)	Structure
I-1	6.11	24031 \pm 4777	
I-2	6.67	122 \pm 18* *determined at 0.3 mg/kg 682 \pm 147** **determined at 1.0 mg/kg	

TABLE 2-continued

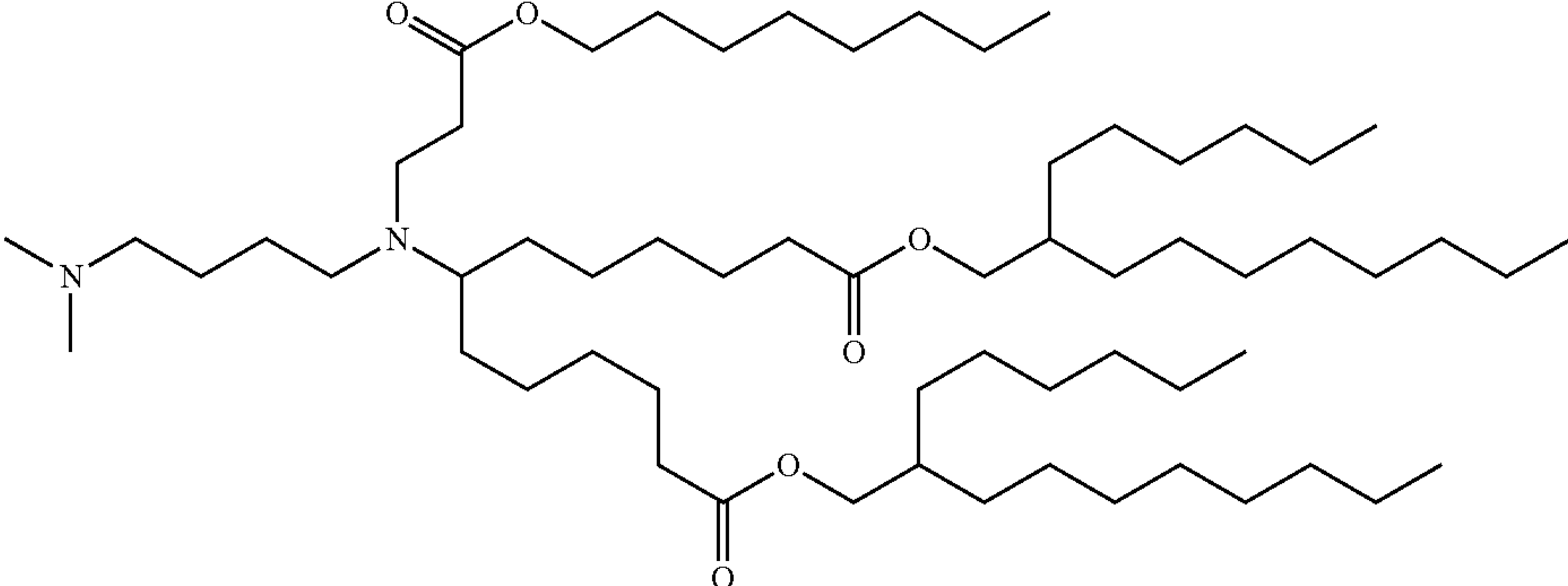
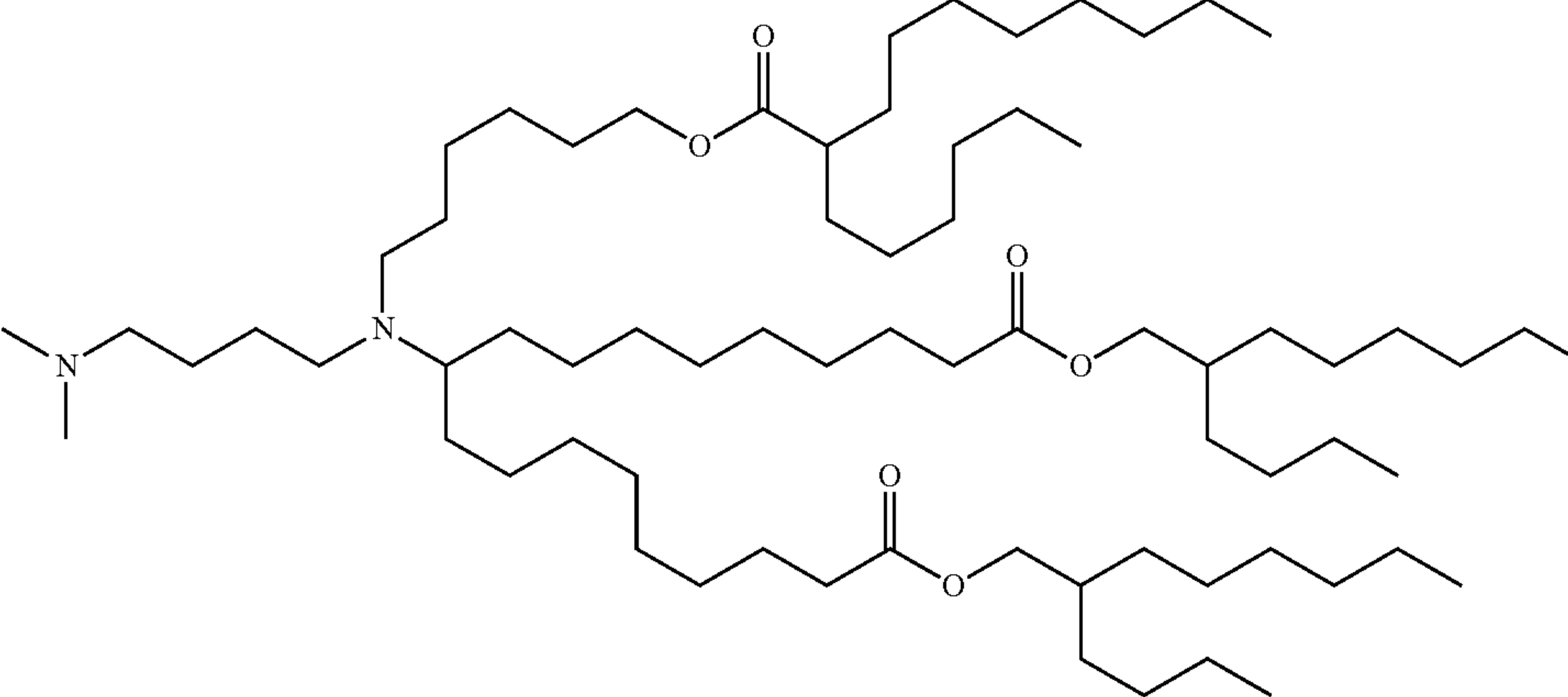
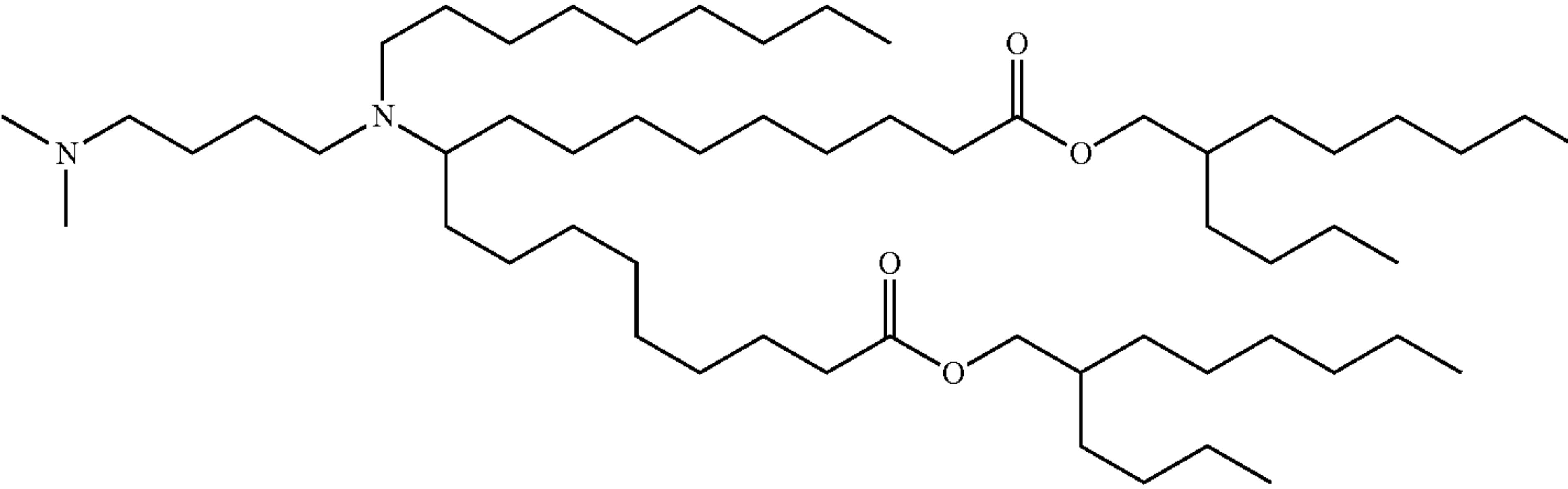
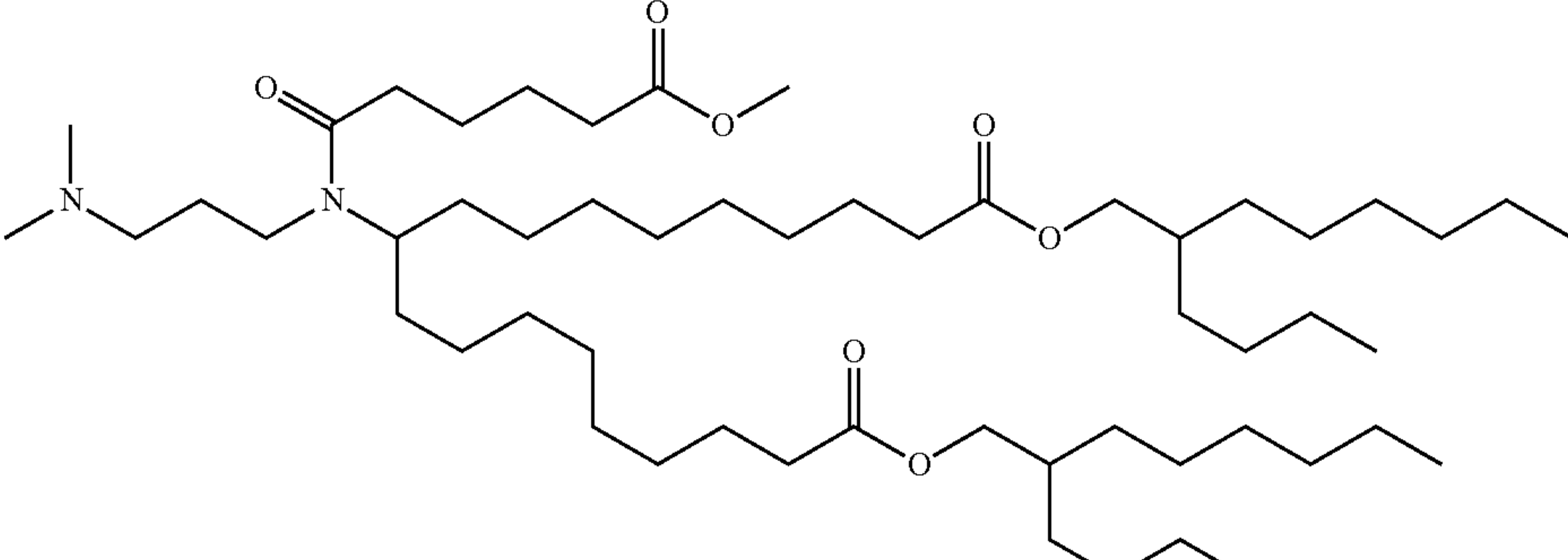
Novel Cationic Lipids and Associated Activity			
Cmp. No.	pKa	Liver Luc @ 1.0 mg/kg (ng luc/g liver)	Structure
I-4	6.17	46932 ± 17399	
I-5	6.08	11720 ± 2439	
I-6	6.22	4093 ± 1036* *determined at 0.3 mg/kg 11290 ± 6455** **determined at 1.0 mg/kg	
I-14	6.80	669 ± 575* *determined at 0.5 mg/kg	

TABLE 2-continued

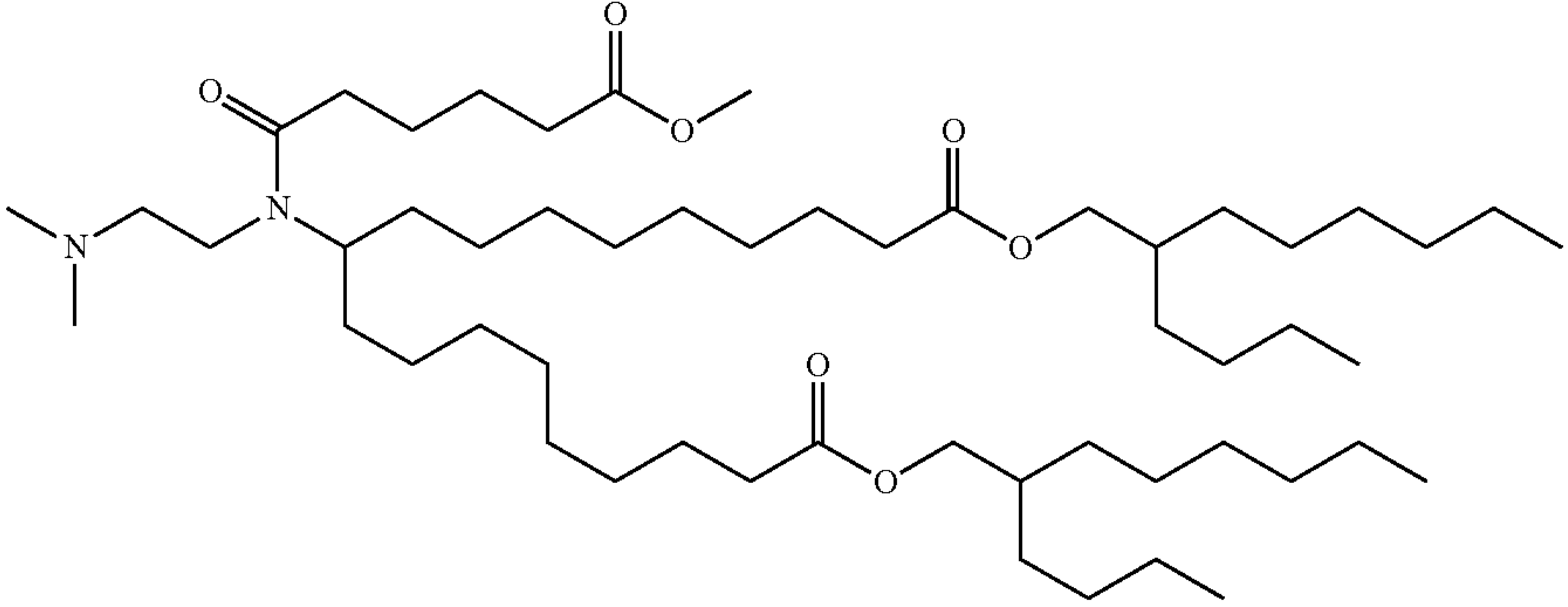
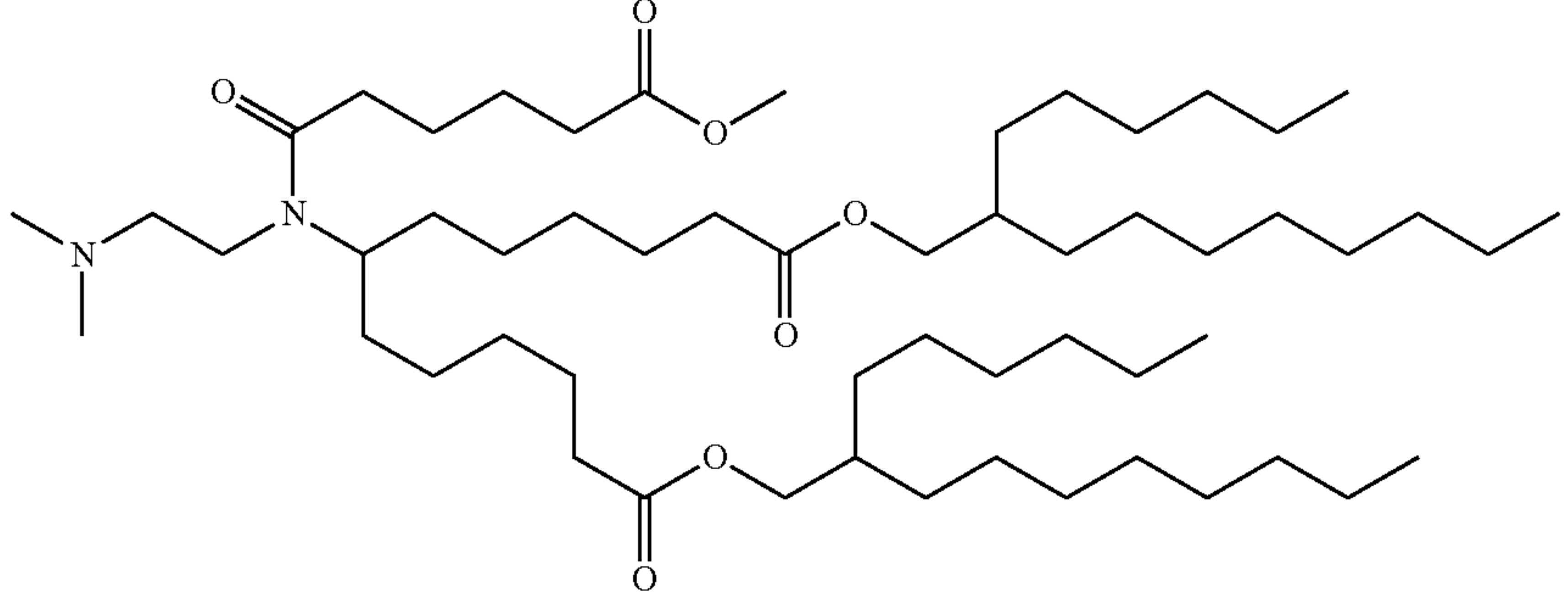
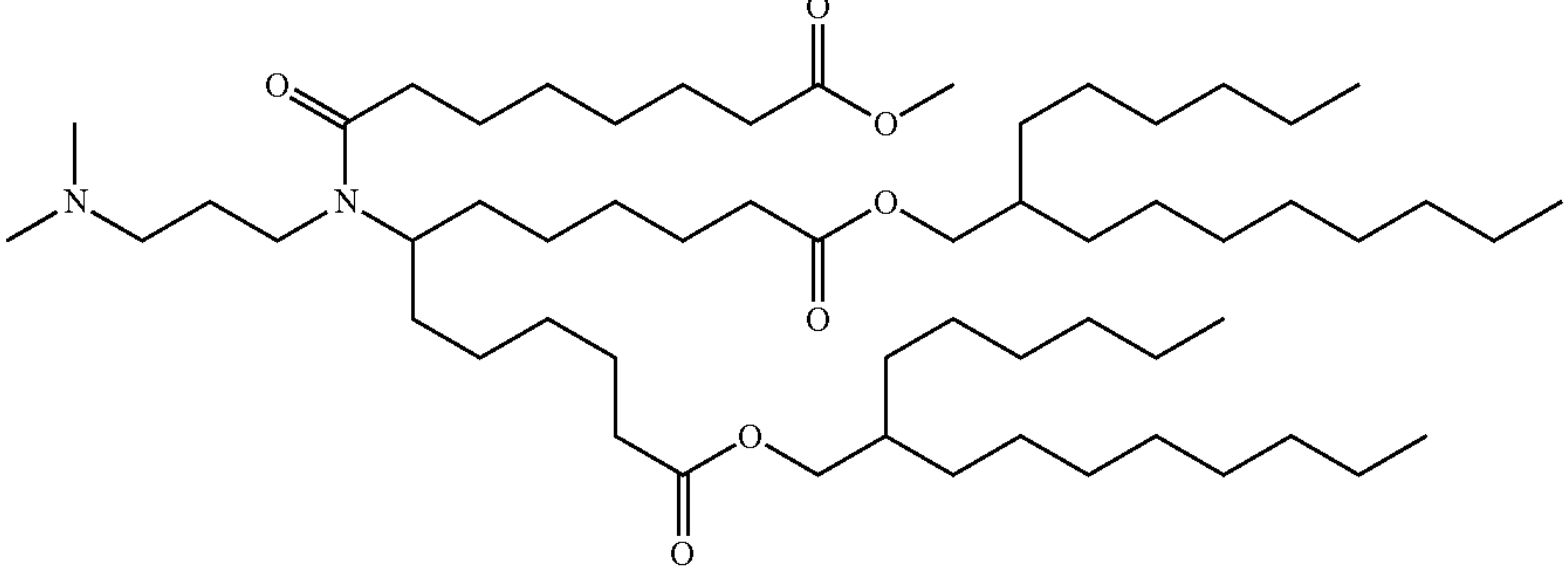
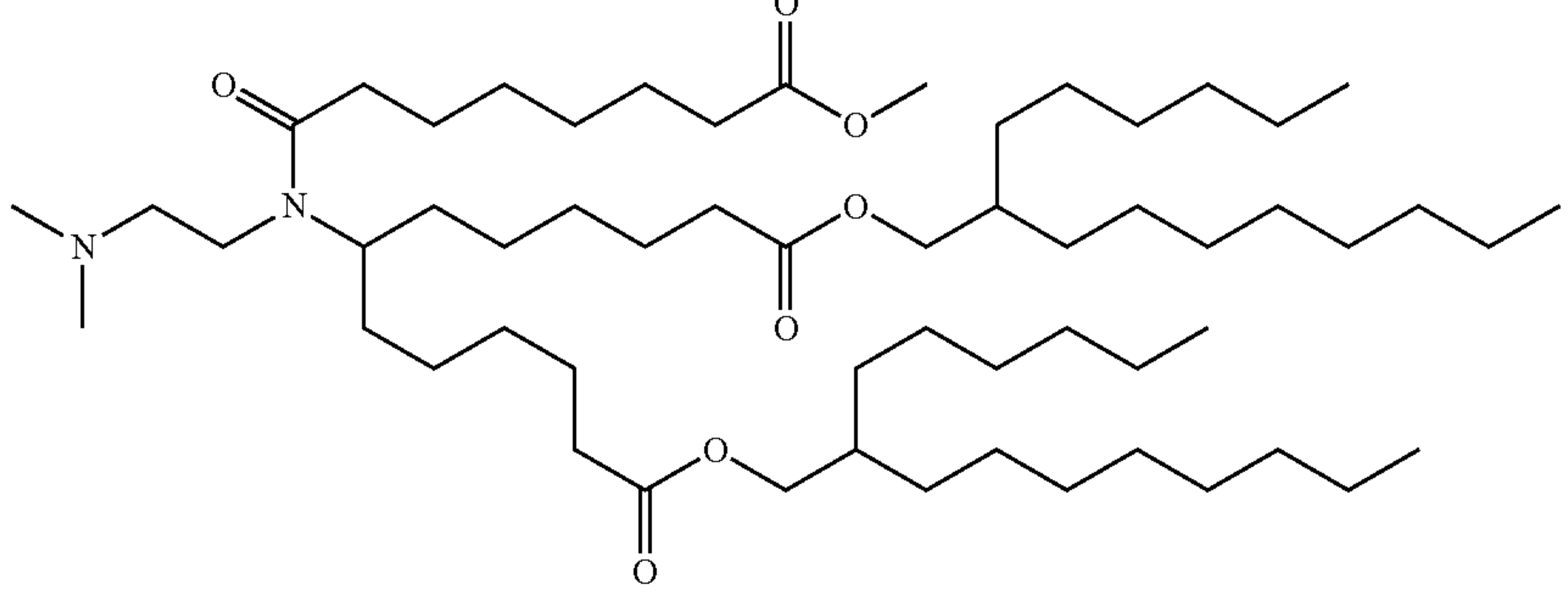
Novel Cationic Lipids and Associated Activity			
Cmp. No.	pKa	Liver Luc @ 1.0 mg/kg (ng luc/g liver)	Structure
I-15	5.96	21357 ± 15325	
I-16	6.25	66763 ± 15823	
I-17	6.78	26691 ± 13186	
I-18	6.02	40650 ± 11479	

TABLE 2-continued

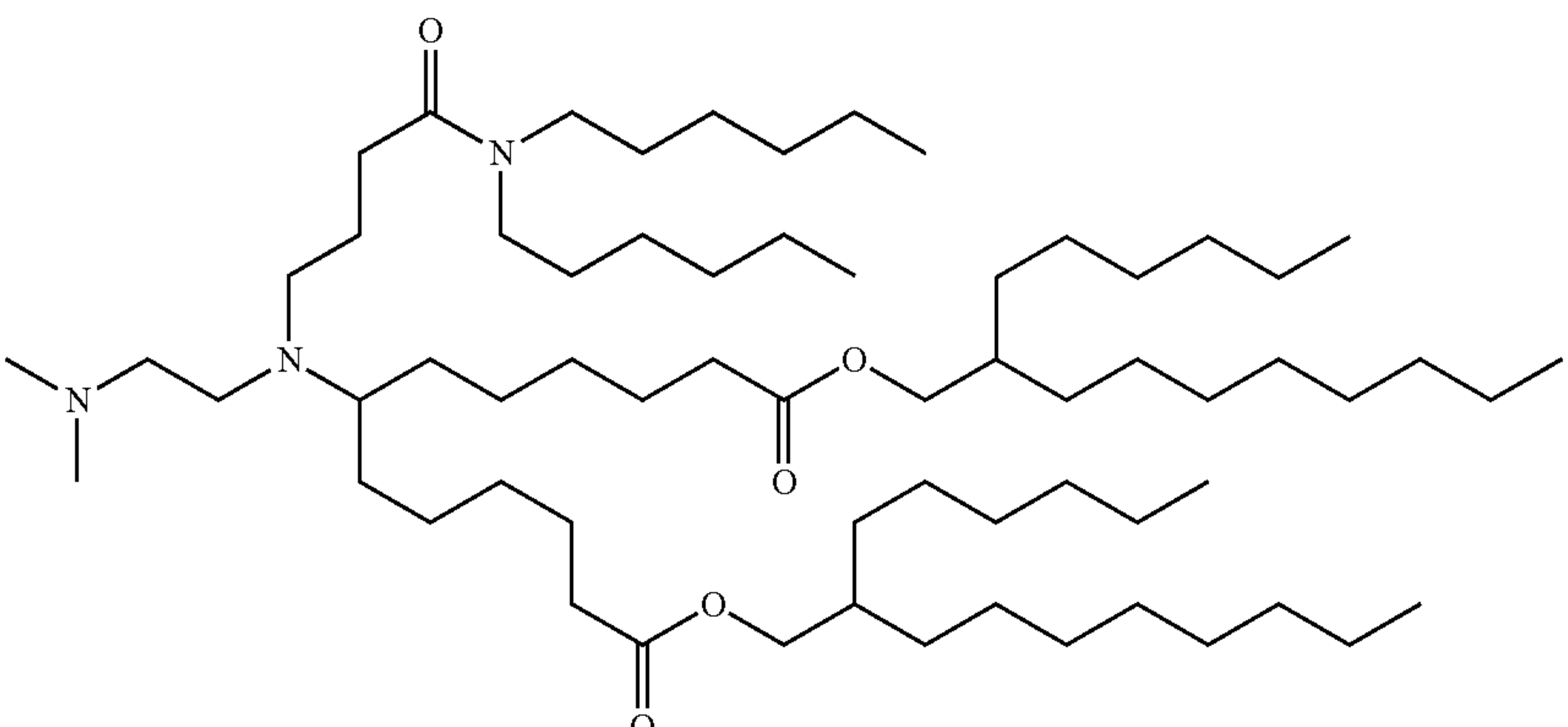
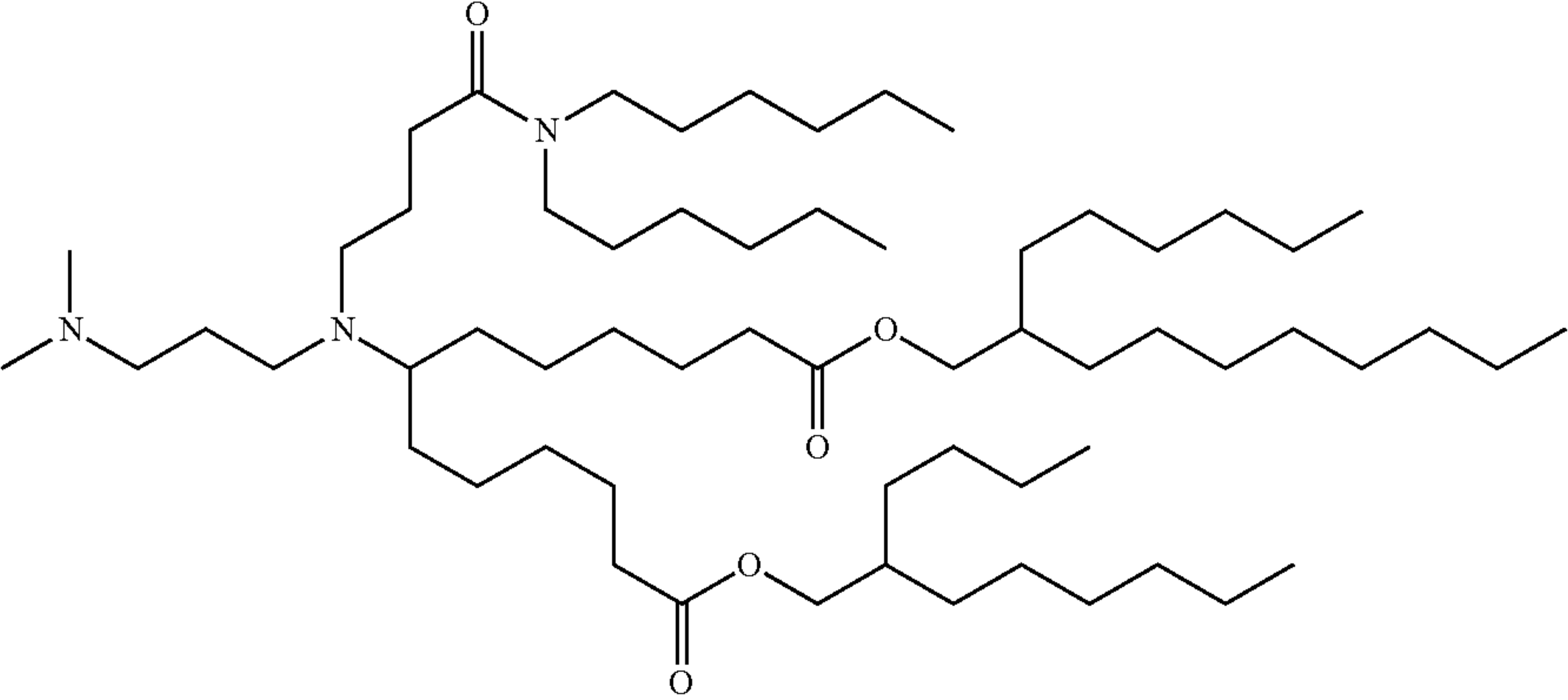
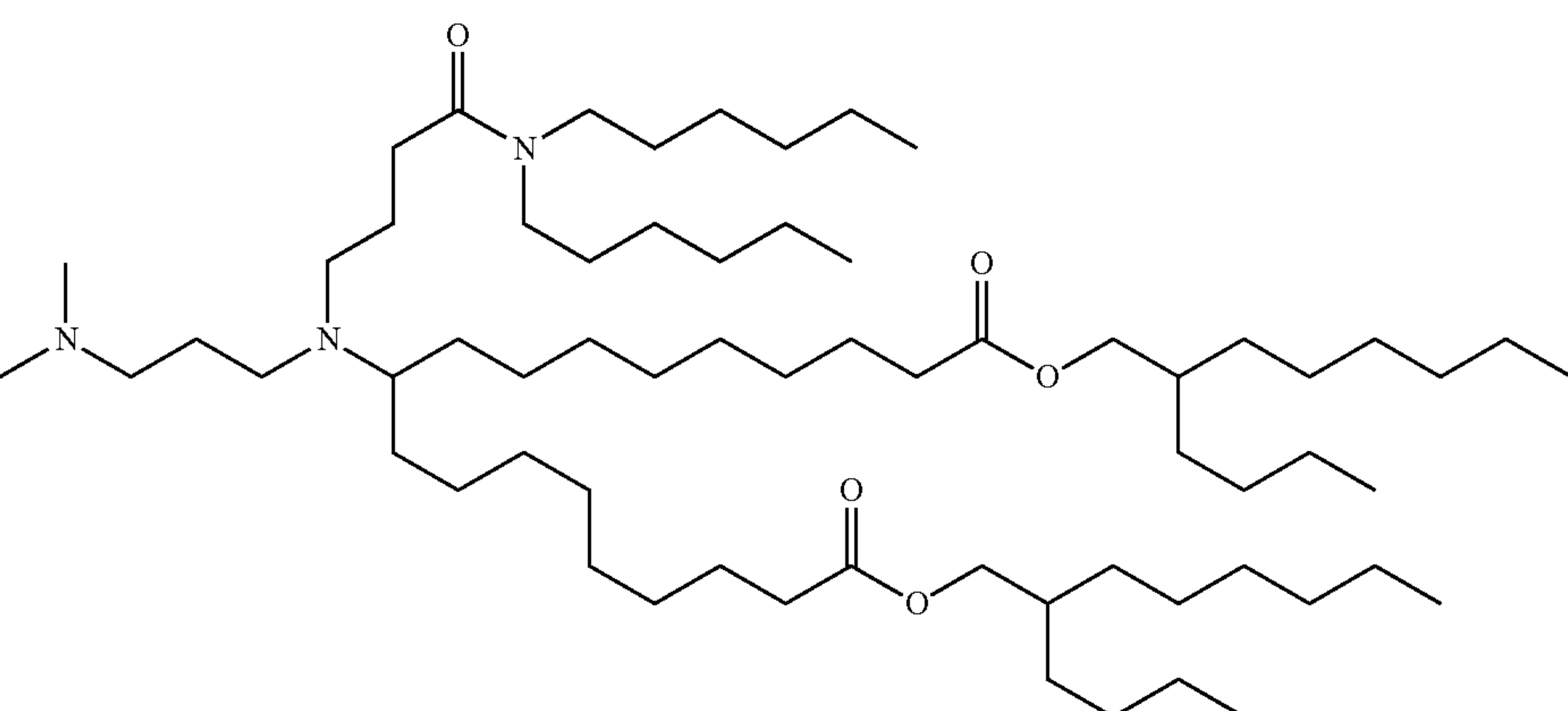
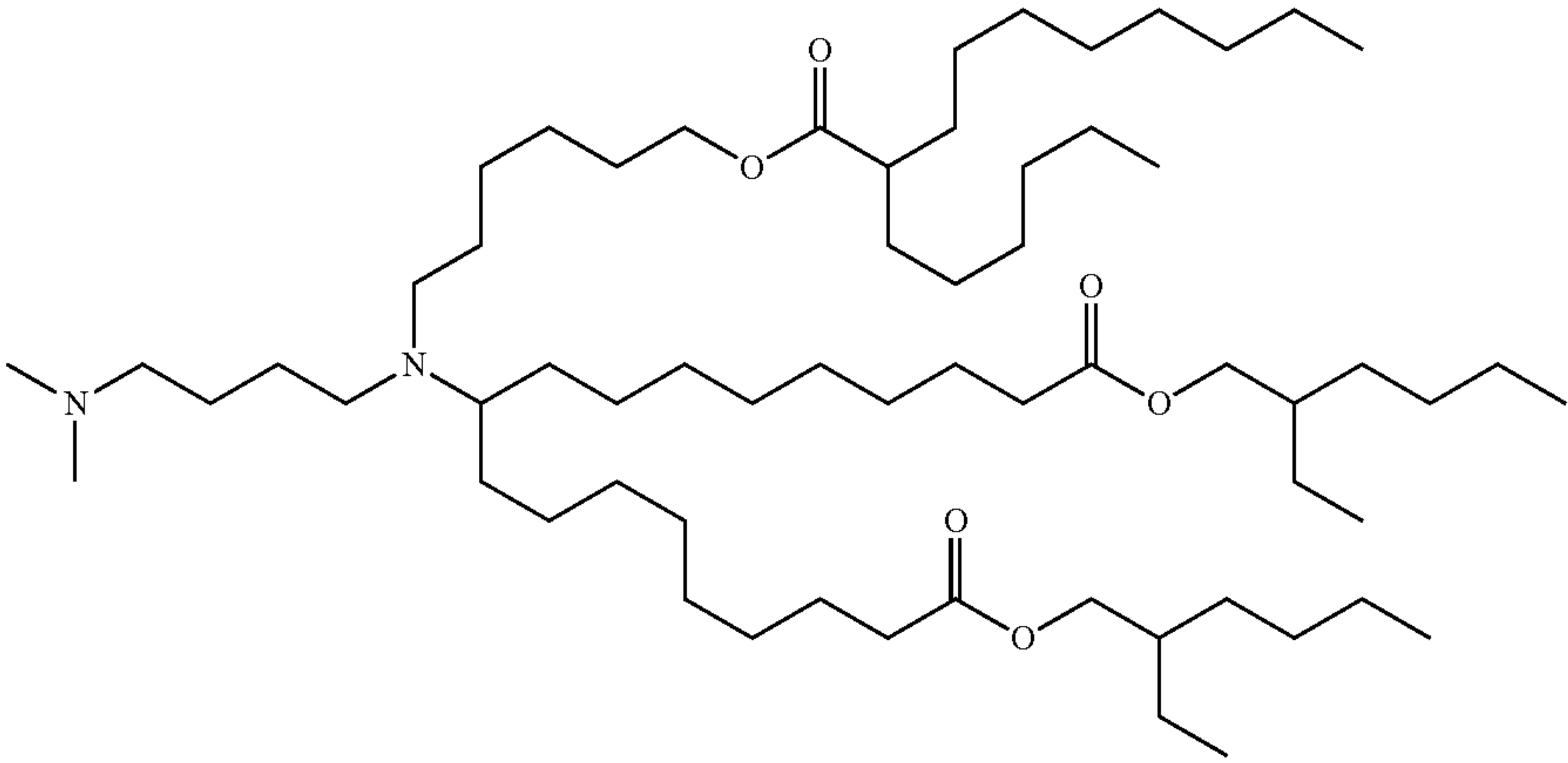
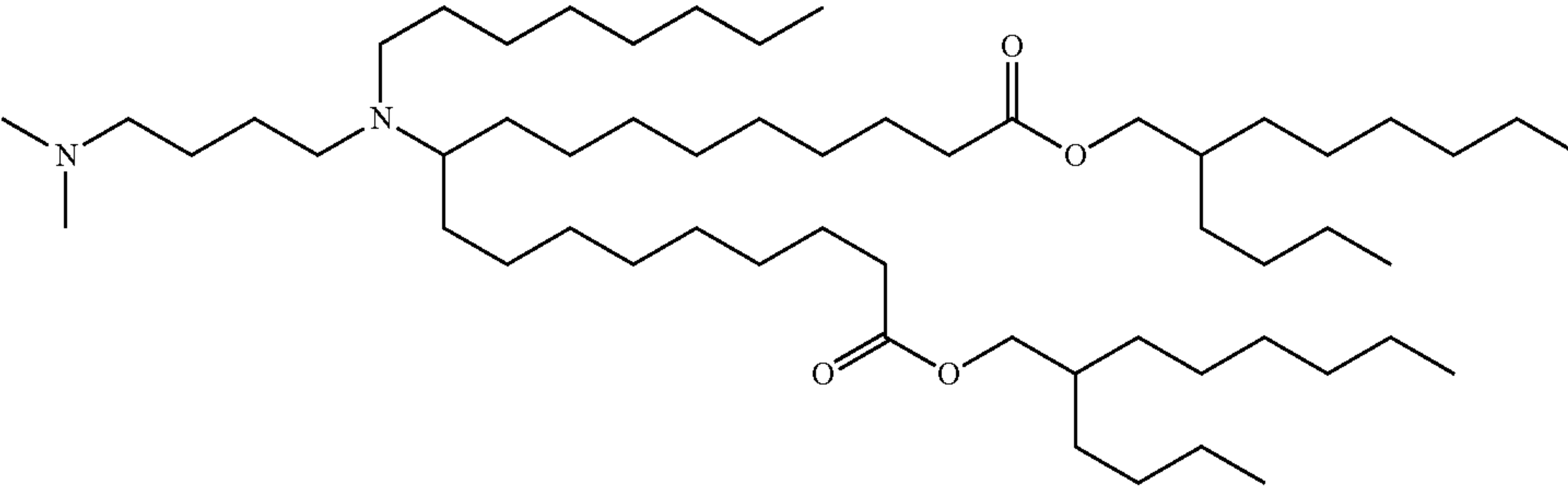
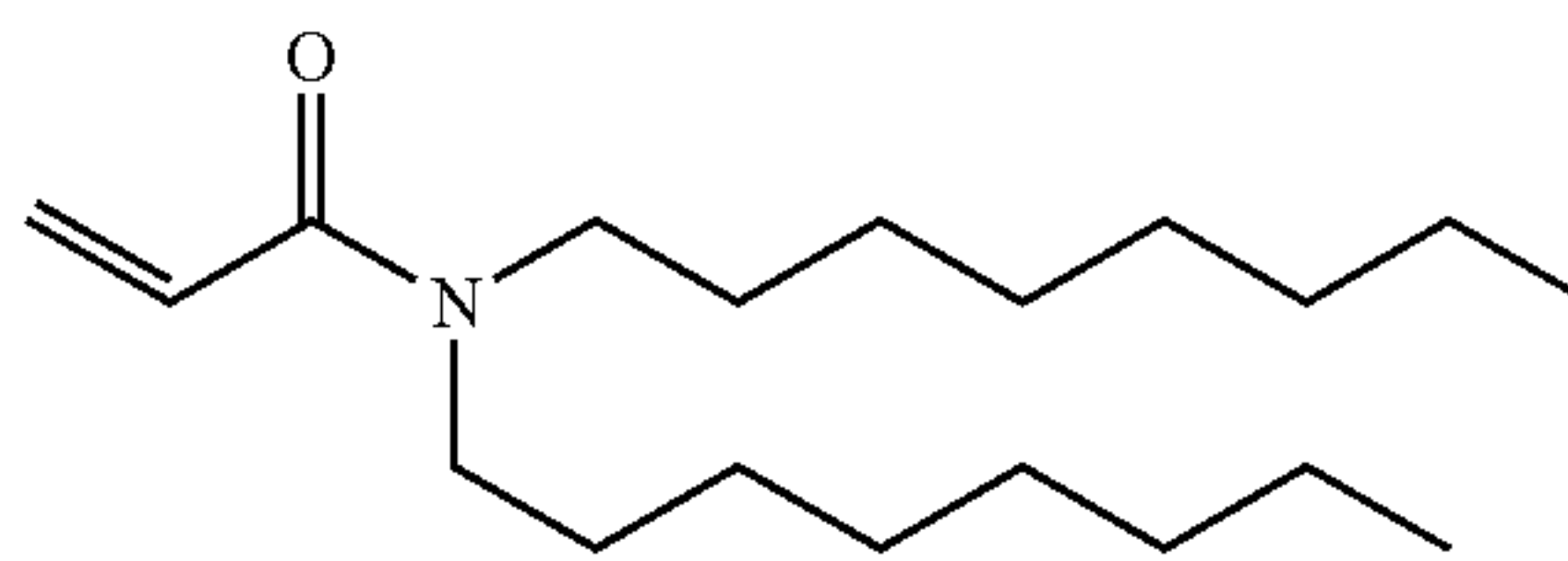
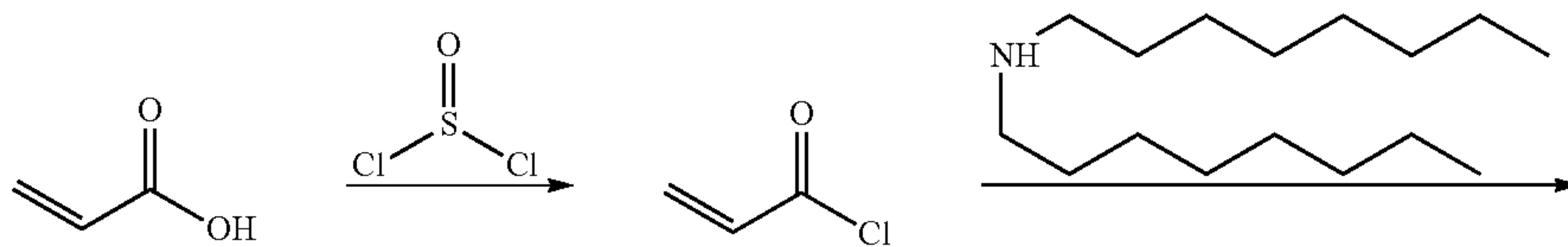
Novel Cationic Lipids and Associated Activity			
Cmp. No.	pKa	Liver Luc @ 1.0 mg/kg (ng luc/g liver)	Structure
I-19	5.95	32706 ± 4621	
I-20	6.48	4140 ± 1117* *determined at 0.3 mg/kg 17095 ± 8181** **determined at 1.0 mg/kg	
I-21	6.40	7723 ± 1714* *determined at 0.3 mg/kg 20223 ± 5982** **determined at 1.0 mg/kg	

TABLE 2-continued

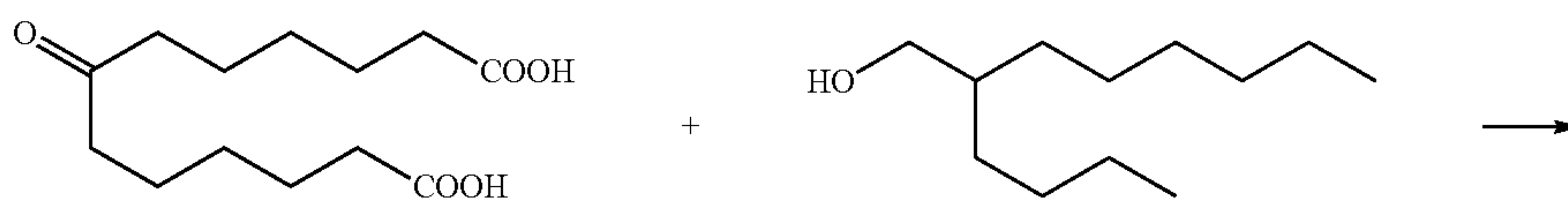
Novel Cationic Lipids and Associated Activity			Structure
Cmp. No.	pKa	Liver Luc @ 1.0 mg/kg (ng luc/g liver)	
I-22	6.28	5291 ± 1348* *determined at 0.3 mg/kg 17654 ± 8167** **determined at 1.0 mg/kg	
I-23	6.33	553 ± 153* *determined at 0.5 mg/kg	

Example 4

Synthesis of bis(2-butyl)octyl 7-((3-(dimethylamino)propyl)(3-(dioctylamino)-3-oxopropyl)amino) tridecanedioate (Compound I-7) 40

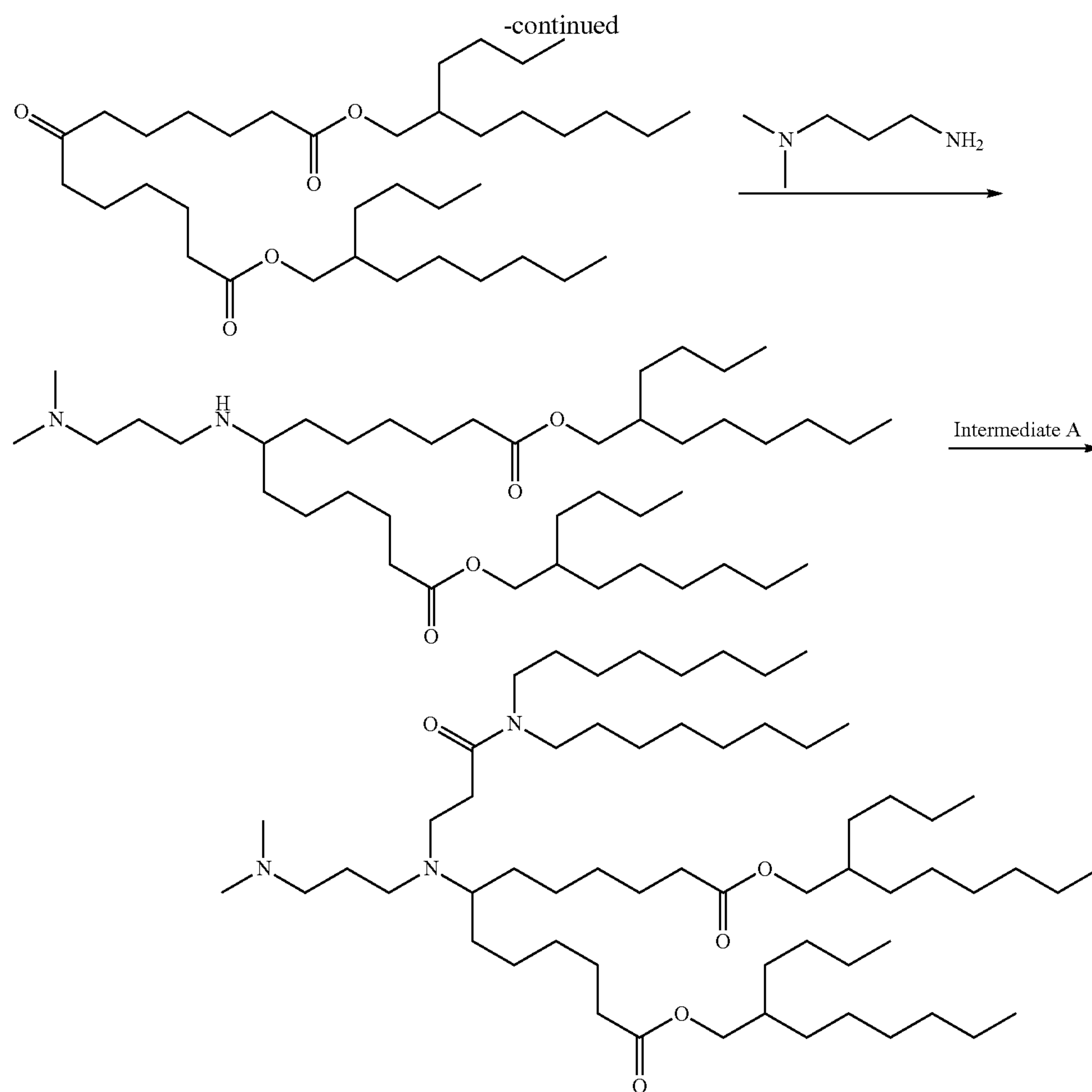


Intermediate A



51

52



I-7

40

Synthesis of Acryloyl Chloride

Acrylic acid (1.20 g, 16.65 mmol) was dissolved in 20 mL anhydrous dichloromethane. Thionyl chloride (1.98 g, 16.65 mmol) was added dropwise while stirring under N_2 , and the reaction mixture was allowed to heat to reflux for 4 hr. After the completion of reaction, the crude product concentrated to yield a pale yellow liquid that used in the next step without further purification.

Synthesis of N,N-Dioctylacrylamide (Intermediate A)

Acryloyl chloride (1.12 g, 12.37 mmol) was added to a cooled solution ($0^\circ C$.) of dioctylamine in dichloromethane with triethylamine (1 equiv) as a base. The reaction mixture was stirred at $0^\circ C$. for 1 h and additional 1 h at room temperature. The reaction mixture was filtered and the solution obtained was washed with hydrochloric acid (1N HCl), then with sat $NaHCO_3$ solution and brine. The solvent evaporated under reduced pressure to yield crude product (colorless liquid) which was used in the following step without further purification.

Synthesis of bis(2-butyloctyl) 7-oxotridecanedioate

To a solution of 2-butyloctane-1-ol (3.85 g, 20.66 mmol), 7-oxotridecanedioic acid (1.34 g, 5.17 mmol) and 4-dim-

ethylaminopyridine (DMAP) (1.9 g, 15.55 mmol) in anhydrous DCM was added DCC (4.27 g, 20.69 mmol). The resulting mixture was allowed to stir overnight at room temperature. The solid (DCU) was then filtered and washed with DCM. The filtrate was concentrated. The residue (oil/solid) was purified by column chromatography on silica gel (0-5% ethyl acetate in hexane). The desired product was obtained as a colorless oil (2.55 g, 42.86 mmol, 83%).

Synthesis of bis(2-butyloctyl) 7-((3-(dimethylamino)propyl)amino)tridecanedioate

A solution of 3-(dimethylamino)-1-propylamine (0.09 g, 0.88 mmol) and bis(2-butyloctyl) 7-oxotridecanedioate (0.37 g, 0.63 mmol) in DCE was treated with sodium triacetoxyborohydride (0.20 g, 0.94 mmol) and AcOH (55 μL , 0.98 mmol) overnight. The solution was washed with dilute aqueous sodium hydroxide solution (1 N NaOH). The organic phase was washed with brine, dried over anhydrous sodium sulfate, filtered and the solvent removed. The residue was passed down a small pad of silica gel, washed with a mixture of DCM/MeOH/ Et_3N (85:15:1). The filtrate was concentrated to give the desired product as a slightly yellow oil (240 mg, 0.35 mmol, 56%).

Synthesis of I-7

An EtOH (10 mL) solution of bis(2-butyloctyl) 7-((3-(dimethylamino)propyl)amino)tridecanedioate (210 mg,

65

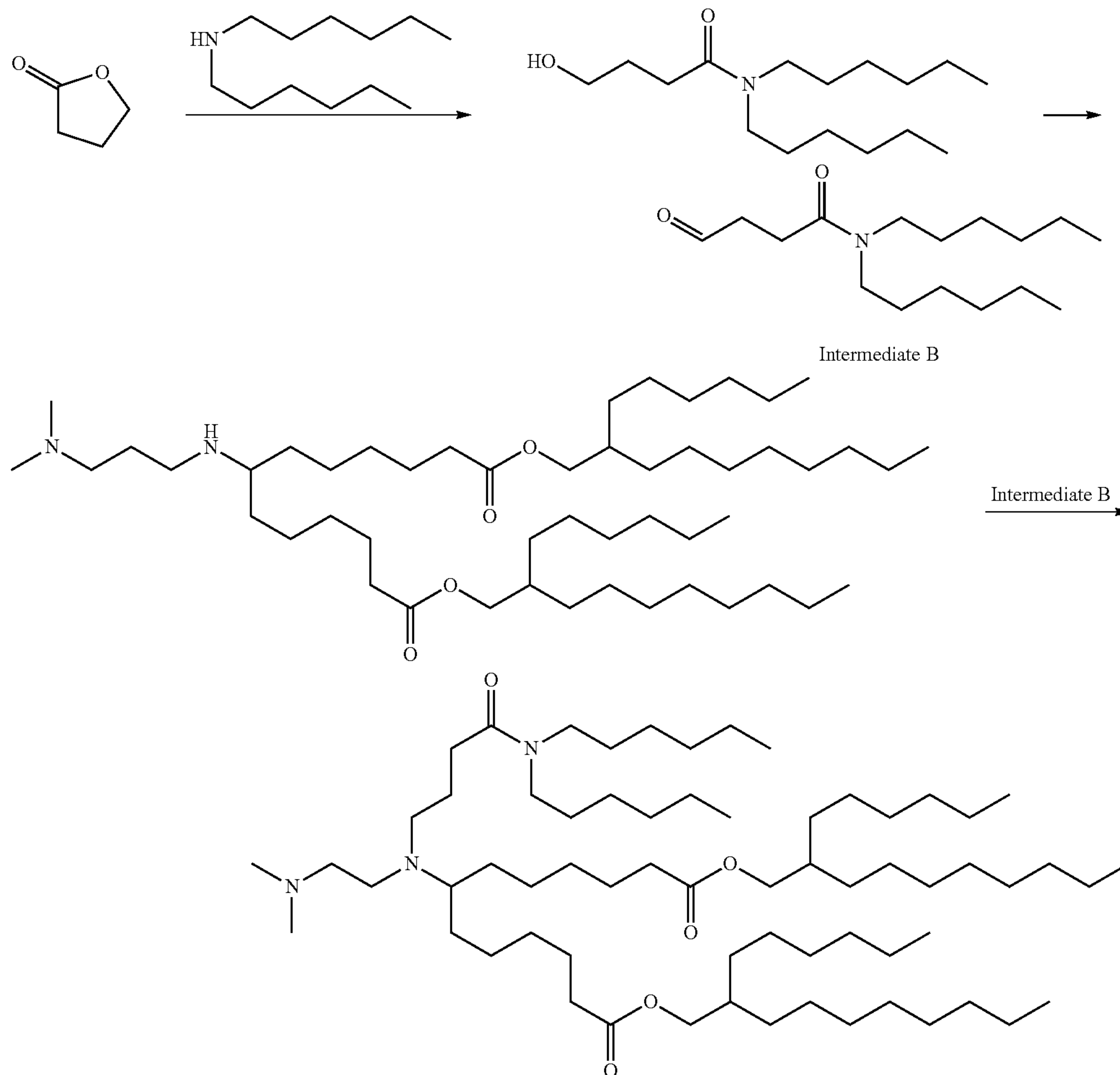
53

0.30 mmol) and N,N-Dioctylacrylamide (1.5 eq, 136 mg, 0.46 mmol) was stirred at room temperature overnight. The reaction mixture was heated to reflux for 7 days. Upon completion of the reaction, the solvent was removed. The residue was dissolved in a mixture of hexanes and EtOAc (19:1) and washed with saturated sodium bicarbonate solution, and brine. The extract was dried over sodium sulfate. The dried extract was filtered through a pad of silica gel. The pad was washed with a mixture of hexane/ethyl acetate/triethylamine (80:20:1). The washing was concentrated to give the crude desired product.

The crude product was further purified by flash dry column chromatography on silica gel (MeOH in chloroform, 0 to 5%). This gave the desired product as a colorless oil (30 mg, 0.03 mmol, 10%). ¹H NMR (400 MHz, CDCl₃) δ: 3.96 (d, 5.8 Hz, 4H), 3.30-3.16 (m, 4H), 2.74 (t, 7.2 Hz, 2H), 2.65-2.24 (m, 17H), 1.80-1.44 (m, 12H), 1.43-1.15 (64H), 0.93-0.82 (m, 18H).

Example 5

Synthesis of bis(2-hexyldecyl) 7-((4-(dihexylamino)-4-oxobutyl)(2-(dimethylamino)ethyl)amino) tridecanedioate (Compound I-19)



I-19

54

Synthesis of N,N-dihexyl-4-oxobutanamide (Intermediate B)

Butyrolactone (2.51 g, 29.15 mmol) and dihexylamine (5.40 g, 29.13 mmol) were heated for 4 days in a pressure flask at 61° C. The reaction mixture was cooled to room temperature. The crude product was purified by column chromatography on silica gel (0% to 5% of MeOH in DCM) to yield N,N-dihexyl-4-hydroxybutanamide as a slightly yellow oil (6.30 g, 79%).

N,N-dihexyl-4-hydroxybutanamide (3.00 g, 11.05 mmol) was dissolved in DCM and treated with pyridinium chlorochromate (2.38 g, 11.05 mmol) for two hours. Diethyl ether was added and the supernatant filtered through silica gel bed. The solvent was removed from the filtrate and resultant oil dissolved in hexane. The suspension was filtered through silica gel bed and the solvent removed. The crude product (colorless liquid) was used in the following step without further purification.

Synthesis of I-19

A solution of N,N-dihexyl-4-oxobutanamide (0.56 g, 1.97 mmol), and bis(2-hexyldecyl) 7-((2-(dimethylamino)ethyl)

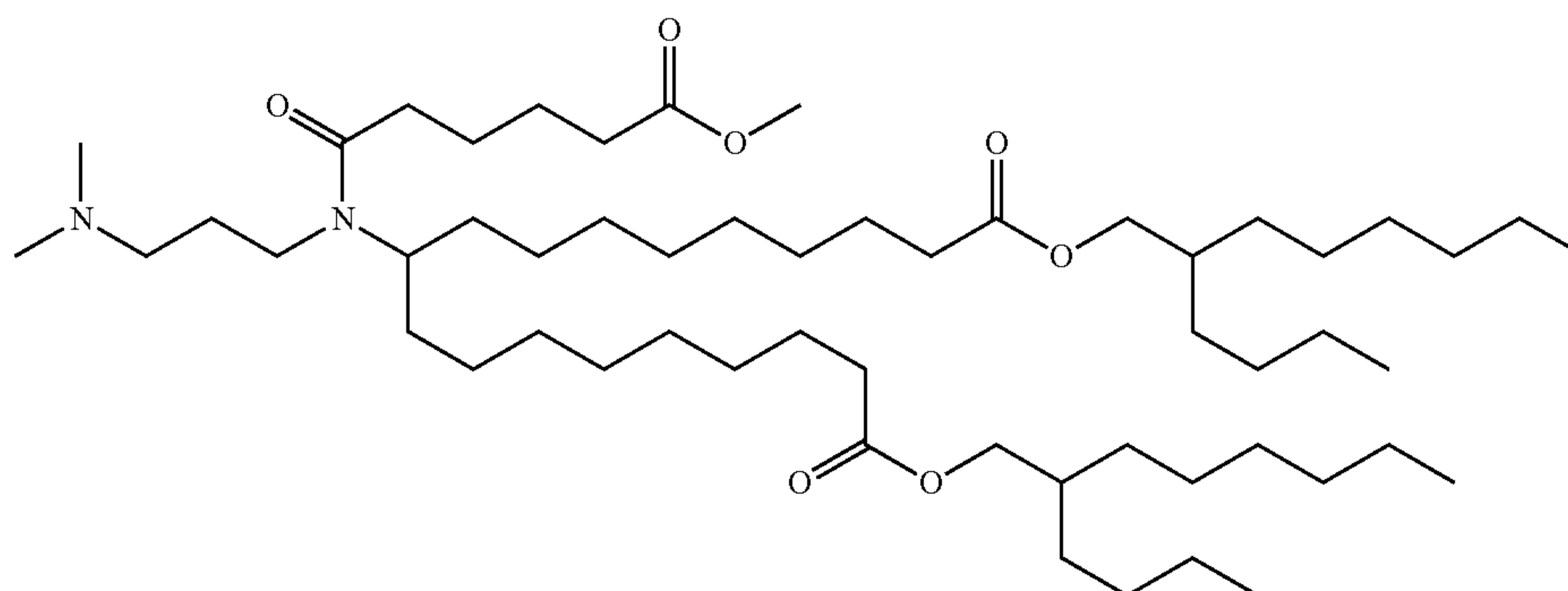
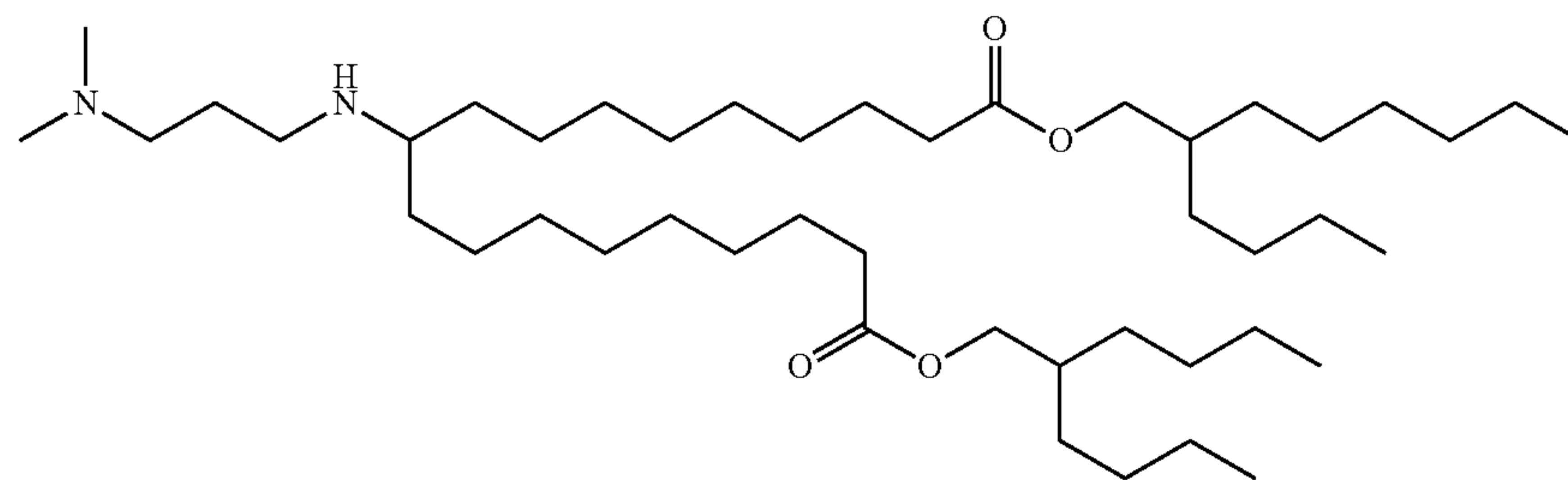
55

amino)tridecanedioate (0.44 g, 0.56 mmol, prepare according to procedures of Example 4) in 1,2-dichloroethane (10 mL) was stirred for 15 min, after which time sodium triacetoxyborohydride (0.41 g, 1.97 mmol) was added in one portion and stirred at room temperature for additional 16 hours. The mixture was concentrated. The residue was taken up in a mixture of hexane and ethyl acetate (96:4) and washed with saturated aqueous NaHCO₃ solution and brine. The organic layer was separated, dried over anhydrous sodium sulphate, filtered and evaporated under reduced pressure to obtain colorless oil. The crude product was purified by flash column chromatography on silica gel (MeOH in chloroform, 0 to 5%) to yield the desired product as a colorless oil (260 mg, 0.25 mmol, 45%). ¹HNMR (400 MHz, CDCl₃) δ: 3.96 (d, 5.8 Hz, 4H), 3.28 (t-like, 7.7 Hz, 2H), 3.20 (t-like, 7.7 Hz, 2H), 2.56-2.47 (m, 2H), 2.44 (t, 6.8 Hz, 2H), 2.39-2.20 (m, 15H), 1.74-1.45 (m, 12H), 1.42-1.15 (72H), 0.93-0.84 (m, 18H).

Example 6

Synthesis of bis(2-butyloctyl) 10-((4-(dihexylamino)-4-oxobutyl)(3-(dimethylamino)propyl)amino)nonadecanedioate (Compound I-21)

Compound I-21 was prepared according to the general procedures of Example 5 to yield 0.05 g of colorless oil, 0.03



I-14

56

mmol, 32%. ¹HNMR (400 MHz, CDCl₃) δ: 3.97 (d, 5.8 Hz, 4H), 3.28 (t-like, 7.6 Hz, 2H), 3.20 (t-like, 7.6 Hz, 2H), 2.43-2.23 (m, 13H), 2.20 (s, 6H), 1.75-1.45 (m, 14H), 1.40-1.12 (m, 68H), 0.93-0.84 (m, 18H).

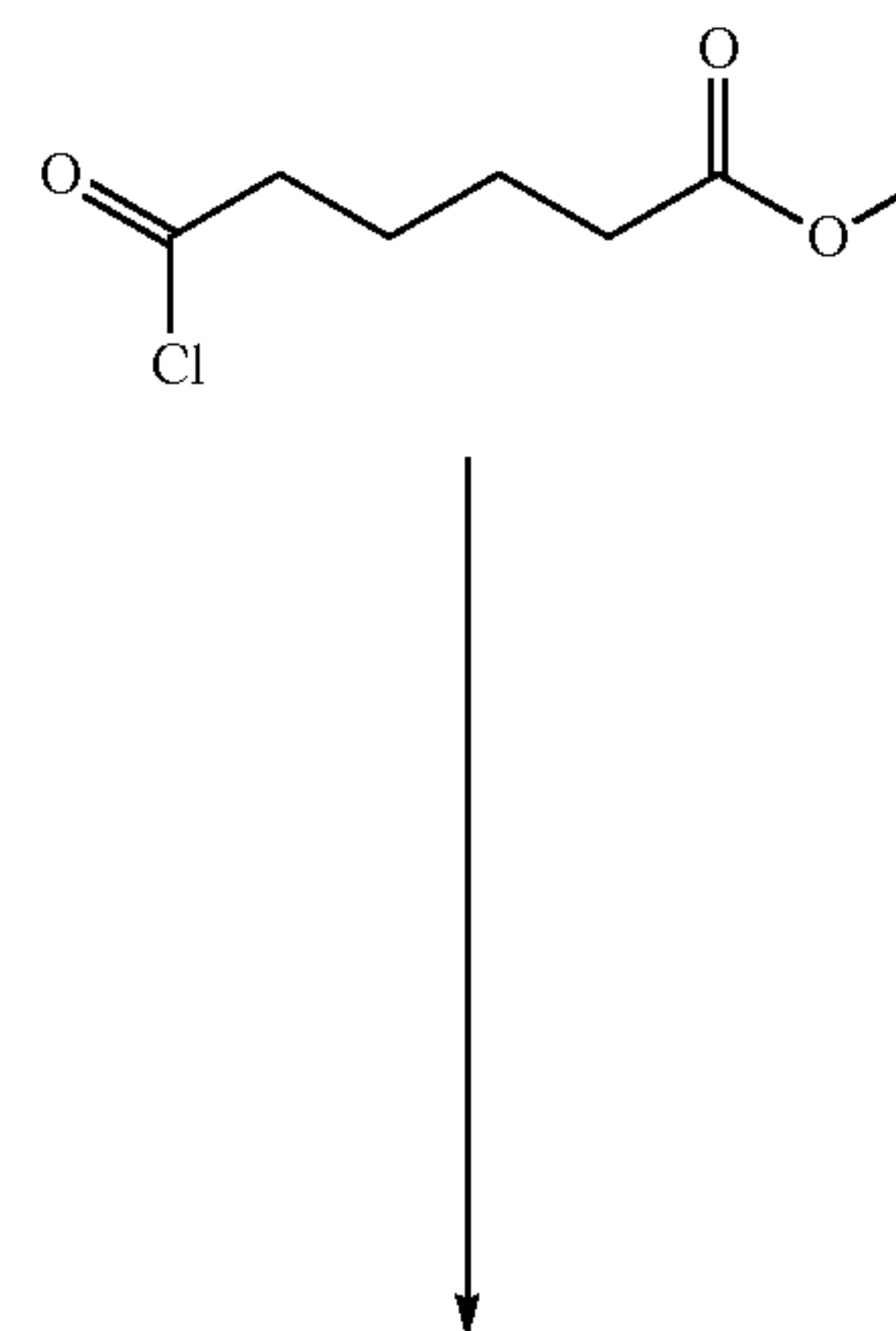
Example 7

Synthesis of bis(2-butyloctyl) 7-((4-(dihexylamino)-4-oxobutyl)(3-(dimethylamino)propyl)amino)tridecanedioate (Compound I-20)

Compound I-20 was prepared according to the general procedures of Example 5 to yield 0.06 g of colorless oil, 0.06 mmol, 41%. ¹HNMR (400 MHz, CDCl₃) δ: 3.96 (d, 5.8 Hz, 4H), 3.27 (t-like, 7.6 Hz, 2H), 3.19 (t-like, 7.6 Hz, 2H), 2.62-2.17 (m, 19H), 1.79-1.43 (m, 14H), 1.42-1.10 (m, 56H), 0.95-0.81 (m, 18H).

Example 8

Synthesis of bis(2-butyloctyl) 10-(N-(3-(dimethylamino)propyl)-6-methoxy-6-oxohexanamido)nonadecanedioate (Compound I-14)



57

Adipoyl chloride (0.12 g, 0.68 mmol) in anhydrous benzene (5 mL) was added via syringe to a solution of bis(2-butyloctyl) 10-((3-(dimethylamino)propyl)amino)nonadecanedioate (0.26 g, 0.34 mmol, prepared according to Example 4), triethylamine (0.3 mL, 2.5 mmol) and DMAP (5 mg) in benzene (10 mL) at RT over the period of 5 min. The mixture was allowed to stir for 2 h and then Methanol (0.5 mL) was added to remove excess acyl chloride. The resulting mixture was stirred for another hour and then filtered through a pad of silica gel, washed with a mixture of hexane/EtOAc/Et₃N (70:30:1) and concentrated. The residue was passed down a silica gel column (0-4% MeOH in DCM gradient), yielding compound I-14 as a colorless oil (0.28 g, 0.30 mmol, 89%). ¹HNMR (400 MHz, CDCl₃) δ: 4.52-4.29 (br., estimated 0.3H, due to slow isomerization about amide bond), 3.96 (d, 5.8 Hz, 4H), 3.65 (s, 3H), 3.59 (quintet-like, 7.0 Hz, 0.7H), 3.14-3.05 (m, 2H), 2.37-2.24 (m, 10H), 2.23-2.18 (m, 6H), 1.73-1.54 (m, 12H), 1.48-1.37 (m, 4H), 1.34-1.14 (m, 52H), 0.93-0.83 (m, 12H).

Example 9

Synthesis of bis(2-butyloctyl) 10-(N-(2-(dimethylamino)ethyl)-6-methoxy-6-oxohexanamido)nonadecanedioate (Compound I-15)

Compound I-15 was prepared according to the general procedures of Example 8, to yield 0.18 g of colorless oil, 0.20 mmol, 85%. ¹HNMR (400 MHz, CDCl₃) δ: 4.53-4.30 (br., 0.3H, due to slow isomerization about amide bond), 3.96 (d, 5.8 Hz, 4H), 3.65 (s, 3H), 3.58 (quintet-like, 7 Hz, 0.7H), 3.27-3.15 (m, 2H), 2.46-2.22 (m, 16H), 1.75-1.54 (m, 10H), 1.50-1.36 (m, 4H), 1.35-1.09 (m, 52H), 0.94-0.82 (m, 12H).

Example 10

Synthesis of bis(2-hexyldecyl) 7-(N-(2-(dimethylamino)ethyl)-6-methoxy-6-oxohexanamido)tridecanedioate (Compound I-16)

Compound I-16 was prepared according to the general procedures of Example 8 to yield 0.27 g of colorless oil, 0.29

58

mmol, 81%. ¹HNMR (400 MHz, CDCl₃) δ: 4.53-4.30 (br., 0.3H, due to slow isomerization about amide bond), 3.99-3.92 (m, 4H), 3.66 (s, 3H), 3.59 (quintet-like, 7.0 Hz, 0.7H), 3.28-3.14 (m, 2H), 2.46-2.20 (m, 16H), 1.75-1.53 (m, 10H), 1.51-1.36 (m, 4H), 1.35-1.09 (m, 56H), 0.94-0.81 (m, 12H).

Example 11

Synthesis of bis(2-hexyldecyl) 7-(N-(3-(dimethylamino)propyl)-8-methoxy-8-oxooctanamido)tridecanedioate (Compound I-17)

Compound I-17 was prepared according to the general procedures of Example 8 to yield 0.08 g of colorless oil, 0.08 mmol, 75%. ¹HNMR (400 MHz, CDCl₃) δ: 4.53-4.30 (br., 0.3H, due to slow isomerization about amide bond), 3.99-3.91 (m, 4H), 3.66 (s, 3H), 3.61 (quintet-like, 7.0 Hz, 0.7H), 3.15-3.06 (m, 2H), 2.34-2.23 (m, 10H), 2.22 (s, 6H), 1.75-1.53 (m, 10H), 1.51-1.38 (m, 4H), 1.37-1.15 (m, 62H), 0.93-0.82 (m, 12H).

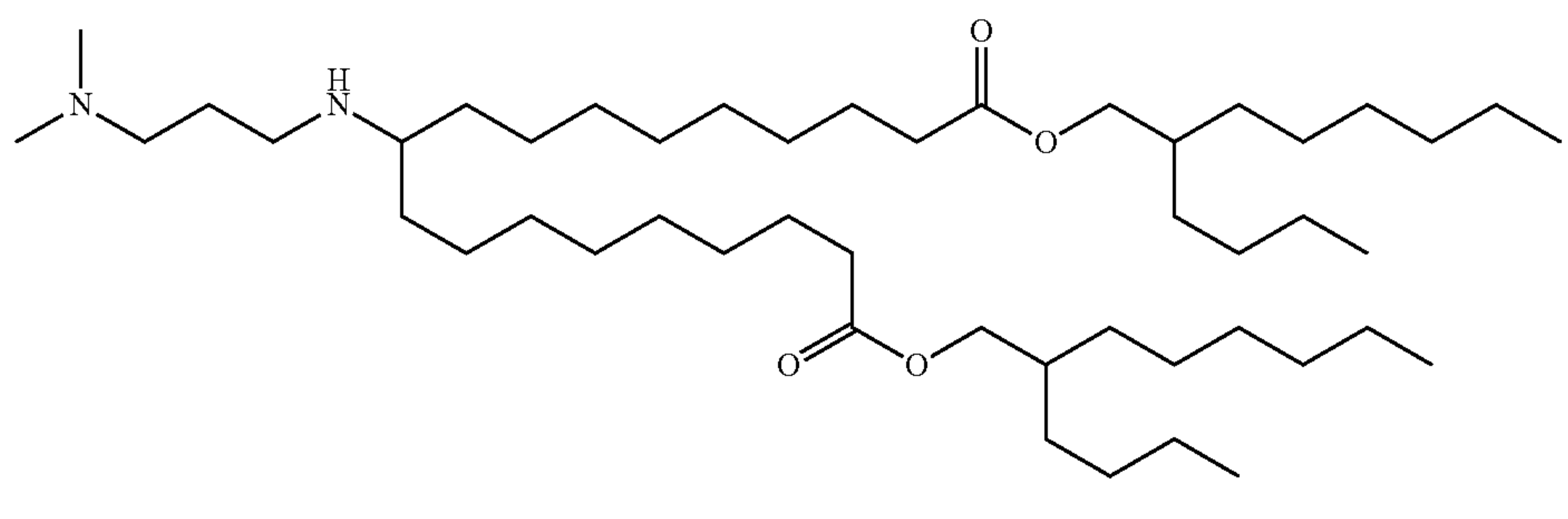
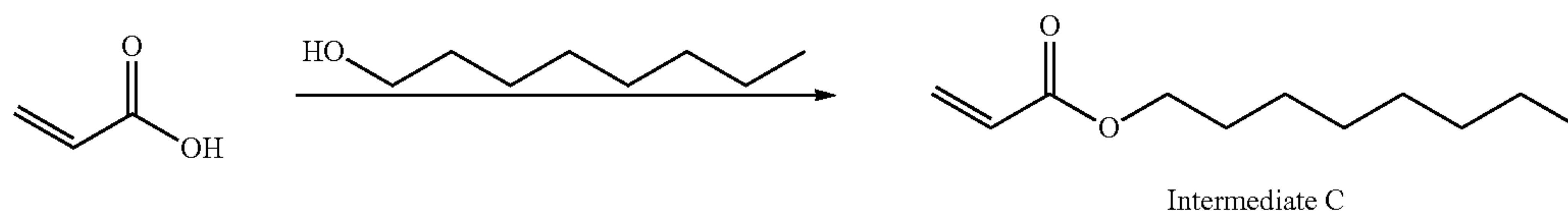
Example 12

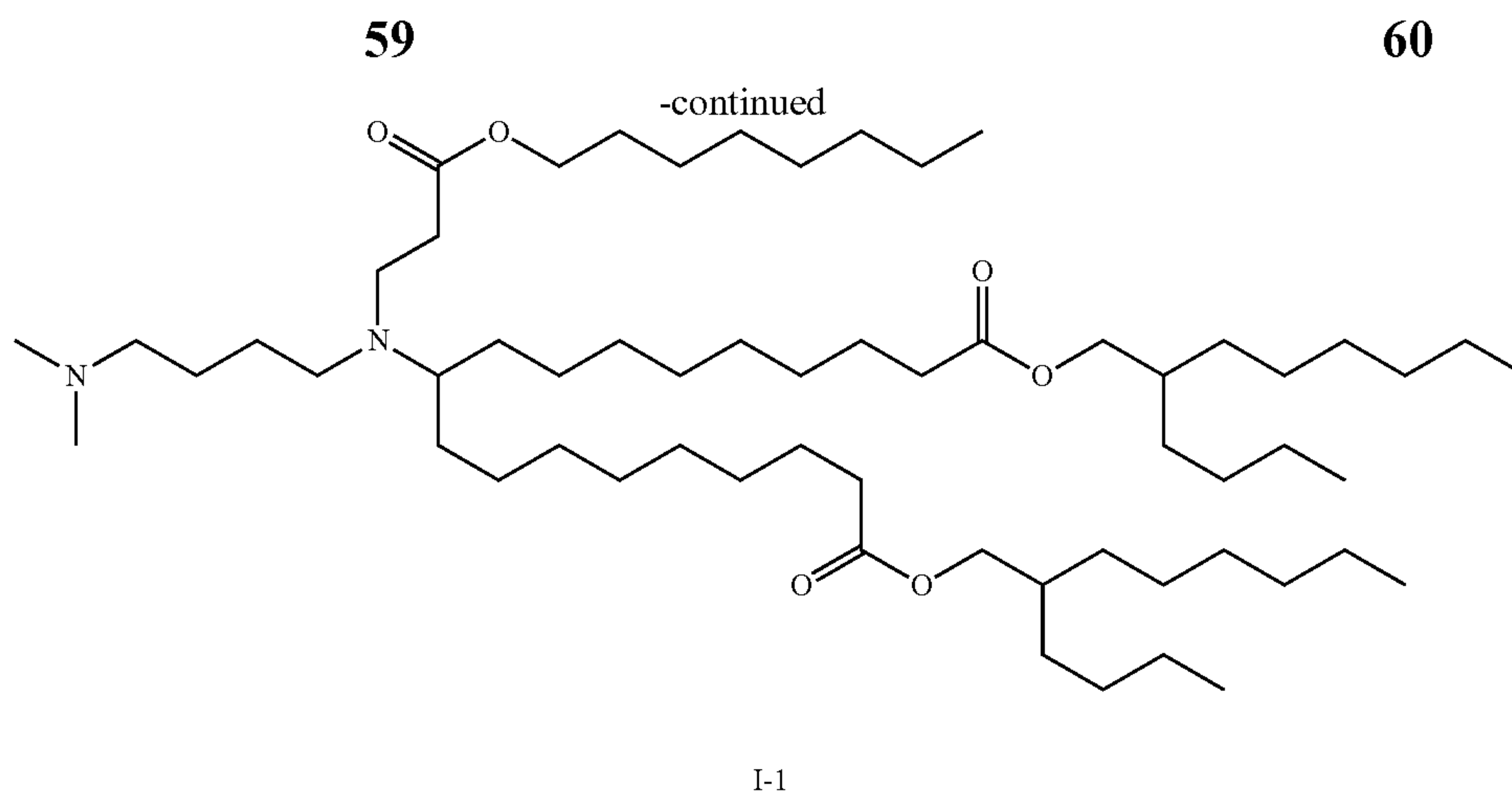
Synthesis of bis(2-hexyldecyl) 7-(N-(2-(dimethylamino)ethyl)-8-methoxy-8-oxooctanamido)tridecanedioate (Compound I-18)

Compound I-18 was prepared according to the general procedures of Example 8 to yield 0.15 g of colorless oil, 0.16 mmol, 79%. ¹HNMR (400 MHz, CDCl₃) δ: 4.53-4.30 (br., 0.3H, due to slow isomerization about amide bond), 3.99-3.92 (m, 4H), 3.66 (s, 3H), 3.61 (quintet-like, 7.0 Hz, 0.7H), 3.26-3.14 (m, 2H), 2.47-2.35 (m, 2H), 2.34-2.20 (m, 14H), 1.73-1.53 (m, 8H), 1.51-1.39 (m, 4H), 1.38-1.14 (m, 62H), 0.93-0.82 (m, 12H).

Example 13

Synthesis of bis(2-butyloctyl) 10-(N-(3-(dimethylamino)propyl)-6-methoxy-6-oxohexanamido)nonadecanedioate (Compound I-1)





Synthesis of Intermediate C

To a solution of acrylic acid (1.1 eq, 8.25 mmol, 594 mg), octanol (1 eq, 975 mg, 7.5 mmol) and DMAP (0.4 eq, 3 mmol, 366 mg) in DCM (15 mL) was added DCC (1.4 eq, 10.5 mmol, 2.16 g). The resulting mixture was stirred at RT for 16 h. The reaction mixture was filtered and the filtrate concentrated. The residue was taken up in hexanes (50 mL) and loaded on a column of silica gel. The column was washed with hexane (40 mL). The fractions were combined, re-loaded on the column and eluted with a mixture of hexane and ethyl acetate (ca 99:1 or 98:2, 200 mL). A colorless oil was obtained (986 mg, 71%).

Synthesis of Compound I-1

An EtOH (10 mL) solution of bis(2-butyloctyl) 10-((4-(dimethylamino)butyl)amino)nonadecanedioate (1 eq., 220 mg, 0.28 mmol, prepared according to general procedures above), and intermediate C (2.75 eq. 0.77 mmol, 140 mg) in a sealed pressure flask was stirred at RT for 4 days under Ar. The reaction mixture was concentrated. The residue was purified twice by flash dry column chromatography on silica gel (hexane-EtOAc-Et₃N, 95:5:0 to 80:20:1 and MeOH in chloroform, 0 to 5%). The desired product was obtained as colorless oil (68 mg, 0.07 mmol, 25%)¹HNMR (400 MHz, CDCl₃) δ: 4.03 (t, 6.9 Hz, 2H), 3.97 (d, 5.8 Hz, 4H), 2.69 (t, 7.2 Hz, 2H), 2.38-2.33 (m, 4H), 2.33-2.26 (m, 1H), 2.29 (t, 7.5 Hz, 4H), 2.26-2.22 (m, 2H), 2.21 (s, 6H), 1.61 (quintet-like, 7.0 Hz, 8H), 1.48-1.08 (70H), 0.92-0.86 (m, 15H).

Example 14

Synthesis of bis(2-butyloctyl) 10-((5-(dimethylamino)pentyl)(3-(octyloxy)-3-oxopropyl)amino)nonadecanedioate (Compound I-2)

Compound I-2 was prepared according to the general procedures of Example 13 to yield 0.05 g of colorless oil, 0.05 mmol, 10%. ¹HNMR (400 MHz, CDCl₃) δ: 4.03 (t, 6.8 Hz, 2H), 3.96 (d, 5.8 Hz, 4H), 2.69 (t, 7.2 Hz, 2H), 2.38-2.21 (m, 17H), 1.61 (quintet-like, 7.0 Hz, 8H), 1.51-1.10 (m, 72H), 0.93-0.84 (m, 15H).

Example 15

Synthesis of bis(2-butyloctyl) 7-((4-(dimethylamino)butyl)(3-(octyloxy)-3-oxopropyl)amino)tridecanedioate (Compound I-3)

Compound I-3 was prepared according to the general procedures of Example 13 to yield 0.01 g of colorless oil, 0.01 mmol, 11%. ¹HNMR (400 MHz, CDCl₃) δ: 4.03 (t, 6.9 Hz, 2H), 3.96 (d, 5.6 Hz, 4H), 2.69 (t, 7.1 Hz, 2H), 2.38-2.20 (m, 17H), 1.69-1.56 (m, 10H), 1.48-1.09 (m, 56H), 0.92-0.84 (m, 15H).

Example 16

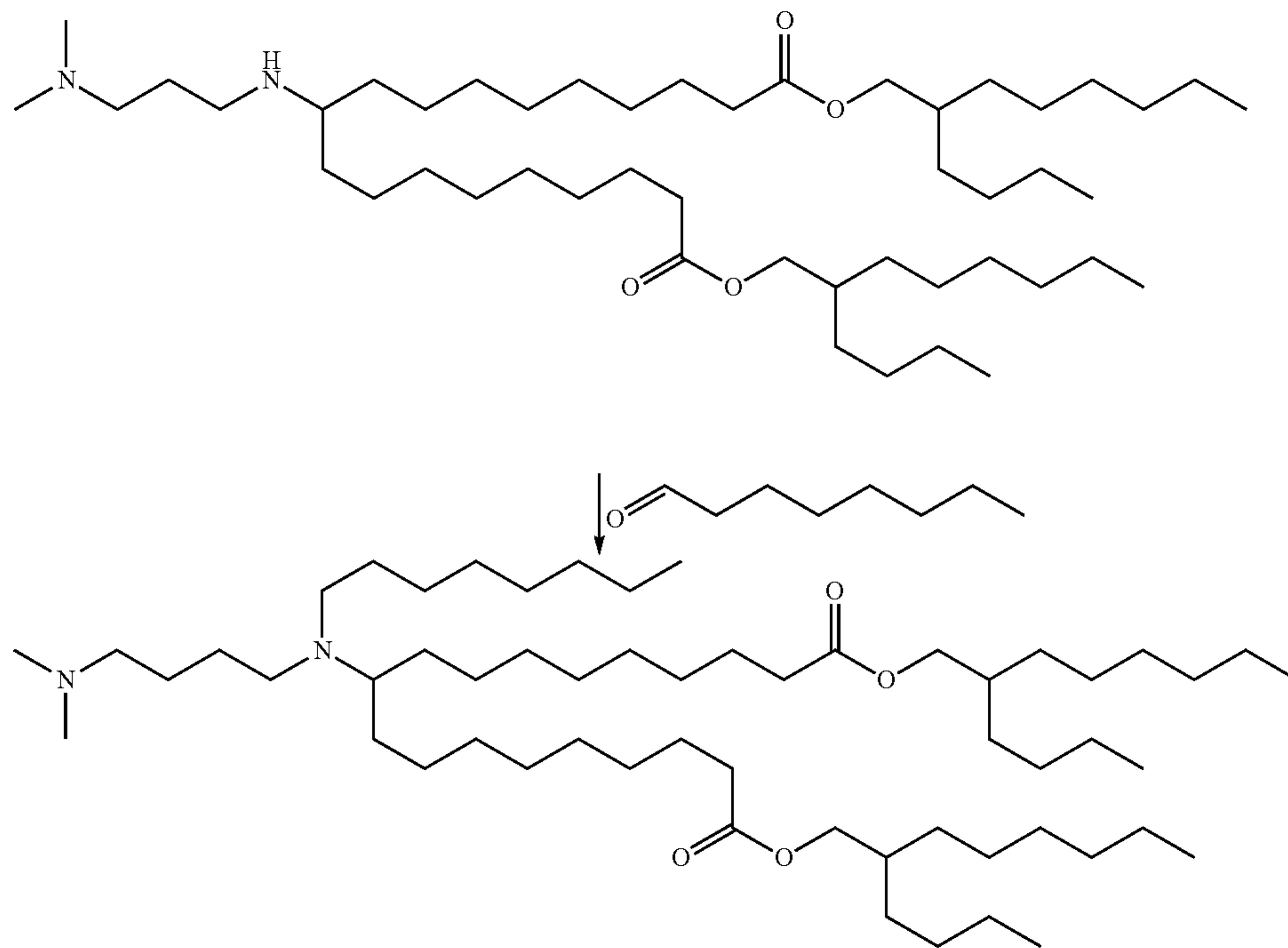
Synthesis of bis(2-hexyldecyl) 7-((4-(dimethylamino)butyl)(3-(octyloxy)-3-oxopropyl)amino)tridecanedioate (Compound I-4)

Compound I-4 was prepared according to the general procedures of Example 13 to yield 0.05 g of colorless oil, 0.05 mmol, 13%. ¹HNMR (400 MHz, CDCl₃) δ: 4.03 (t, 6.8 Hz, 2H), 3.96 (d, 5.8 Hz, 4H), 2.69 (t, 7.1 Hz, 2H), 2.39-2.21 (m, 17H), 1.66-1.09 (m, 82H), 0.88 (t, 7.0 Hz, 15H).

61

Example 17

Synthesis of bis(2-butyloctyl) 10-((4-(dimethylamino)butyl)(octyl)amino)nonadecanedioate (Compound I-23)



I-23

Synthesis of Compound I-23

A solution of octanal (3.5 eq, 0.90 mmol, 115 mg, 0.141 mL) and bis(2-butyloctyl) 10-((4-(dimethylamino)butyl) amino)nonadecanedioate (200 mg, 0.26 mmol, prepared according to general procedures above) in 1,2-dichloroethane (5 mL) was stirred for 15 min, after which time sodium triacetoxyborohydride (3.5 eq, 0.9 mmol, 190 mg) was added in one portion. Stirring was continued at RT for 16 hours. The reaction mixture was concentrated. The residue was purified twice by flash dry column chromatography on silica gel (hexane-EtOAc-Et₃N, 95:5:0 to 80:20:1 and MeOH in chloroform, 0 to 5%). The desired product was obtained as colorless oil (203 mg, 0.23 mmol, 88%). ¹HNMR (400 MHz, CDCl₃) δ: 3.97 (d, 5.8 Hz, 4H), 2.40-2.18 (m, 17H), 1.69-1.56 (m, 6H), 1.52-1.10 (m, 72H), 0.92-0.86 (m, 15H).

Example 18

Synthesis of bis(2-ethylhexyl) 10-((4-(dimethylamino)butyl)(6-((2-hexyldecanoyl)oxy)hexyl)amino) nonadecanedioate (Compound I-22)

Compound I-22 was prepared according to the general procedures of Example 17 to yield 0.19 g of colorless oil, 0.19 mmol, 80%. ¹HNMR (400 MHz, CDCl₃) δ: 4.06 (t, 6.7 Hz, 2H), 3.97 (d, 5.6 Hz, 4H), 2.39-2.26 (m, 11H), 2.23 (s, 6H), 1.68-1.10 (m, 83H), 0.94-0.82 (m, 18H).

62

Example 19

Synthesis of bis(2-butyloctyl) 10-((4-(dimethylamino)butyl)(6-((2-hexyldecanoyl)oxy)hexyl)amino) nonadecanedioate (Compound I-5)

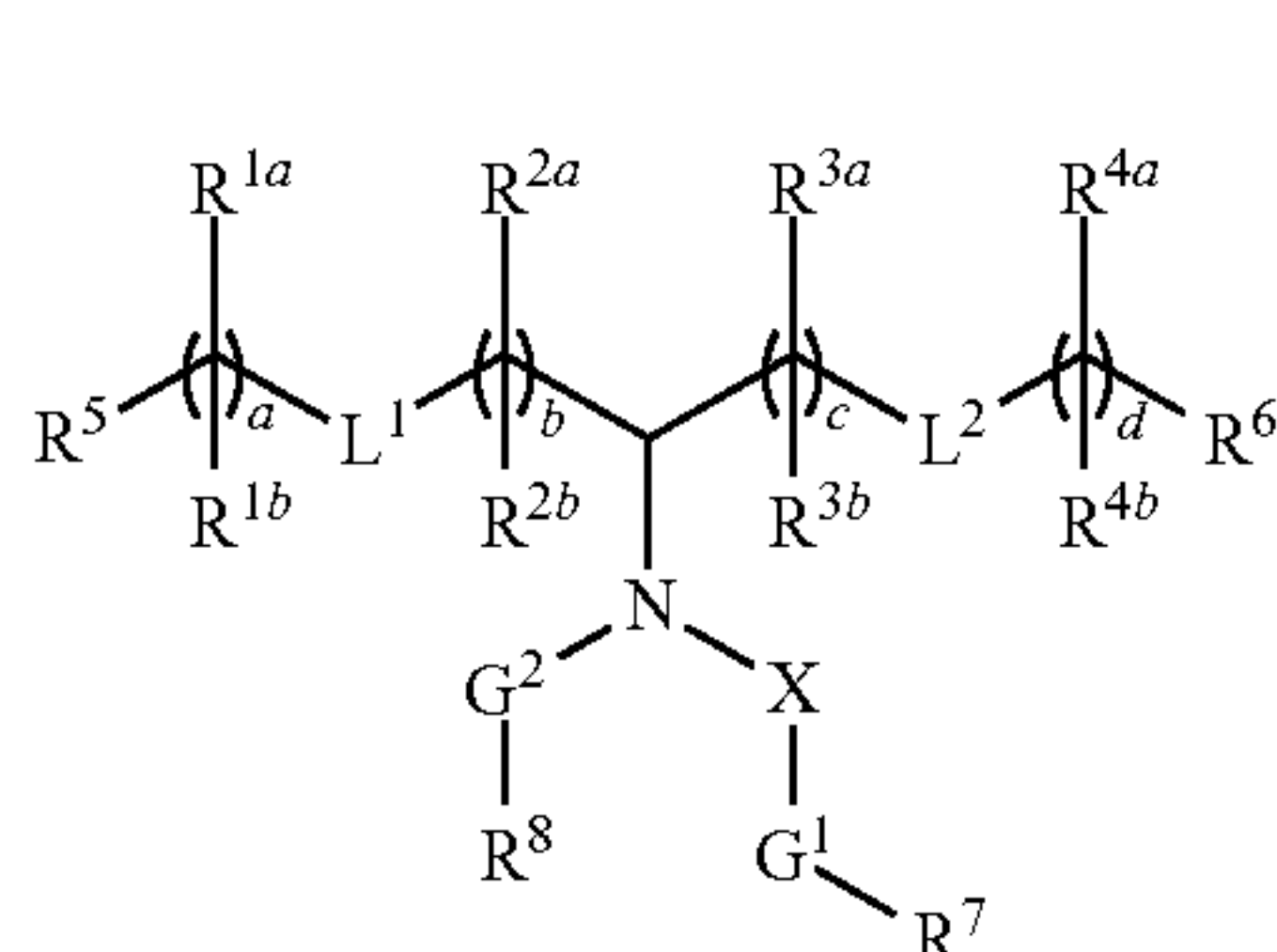
Compound I-5 was prepared according to the general procedures of Example 17 to yield 0.04 g of colorless oil, 0.04 mmol, 73%. ¹HNMR (400 MHz, CDCl₃) δ: 4.05 (t, 7.0 Hz, 2H), 3.96 (d, 5.8 Hz, 4H), 2.38-2.26 (m, 11H), 2.23 (s, 6H), 1.71-1.09 (m, 99H), 0.95-0.82 (m, 18H).

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments. These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

63

The invention claimed is:

1. A compound having a structure of Formula (I):



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof, wherein:

G^1 and G^2 are each independently C_1 - C_6 alkylene;

L^1 and L^2 are each independently $-O(C=O)-$ or $-(C=O)O-$;

R^{1a} and R^{1b} are, at each occurrence, independently either:

(a) H or C_1 - C_{12} alkyl; or (b) R^{1a} is H or C_1 - C_{12} alkyl, and R^{1b} together with the carbon atom to which it is bound is taken together with an adjacent R^{1b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{2a} and R^{2b} are, at each occurrence, independently either:

(a) H or C_1 - C_{12} alkyl; or (b) R^{2a} is H or C_1 - C_{12} alkyl, and R^{2b} together with the carbon atom to which it is bound is taken together with an adjacent R^{2b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{3a} and R^{3b} are, at each occurrence, independently either

(a): H or C_1 - C_{12} alkyl; or (b) R^{3a} is H or C_1 - C_{12} alkyl, and R^{3b} together with the carbon atom to which it is bound is taken together with an adjacent R^{3b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^{4a} and R^{4b} are, at each occurrence, independently either:

(a) H or C_1 - C_{12} alkyl; or (b) R^{4a} is H or C_1 - C_{12} alkyl, and R^{4b} together with the carbon atom to which it is bound is taken together with an adjacent R^{4b} and the carbon atom to which it is bound to form a carbon-carbon double bond;

R^5 and R^6 are each independently H or methyl;

R^7 is $-O(C=O)R^{10}$, $-(C=O)OR^{10}$, $-NR^9(C=O)R^{10}$ or $-(C=O)NR^9R^{10}$;

R^8 is OH, $-N(R^{11})(C=O)R^{12}$, $-(C=O)NR^{11}R^{12}$, $-NR^{11}R^{12}$, $-(C=O)OR^{12}$ or $-O(C=O)R^{12}$;

R^9 is H or C_1 - C_{15} alkyl;

R^{10} is C_1 - C_{15} alkyl;

R^{11} is H or C_1 - C_6 alkyl;

R^{12} is C_1 - C_6 alkyl;

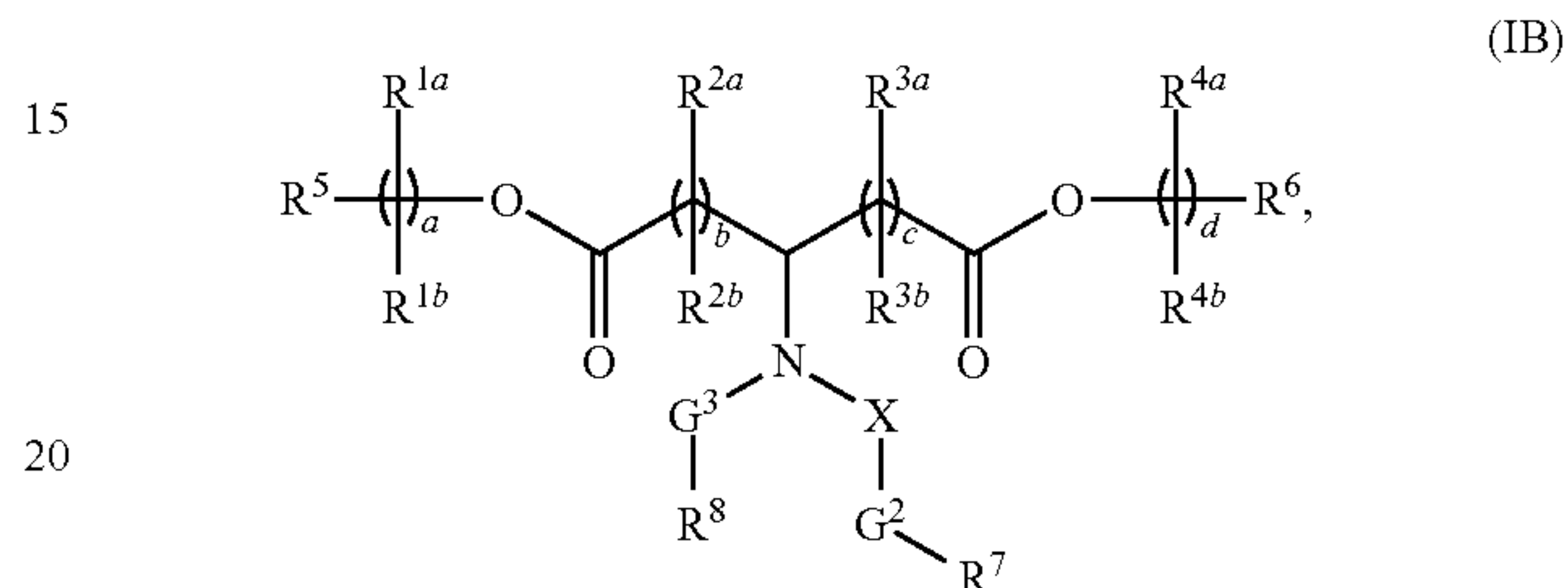
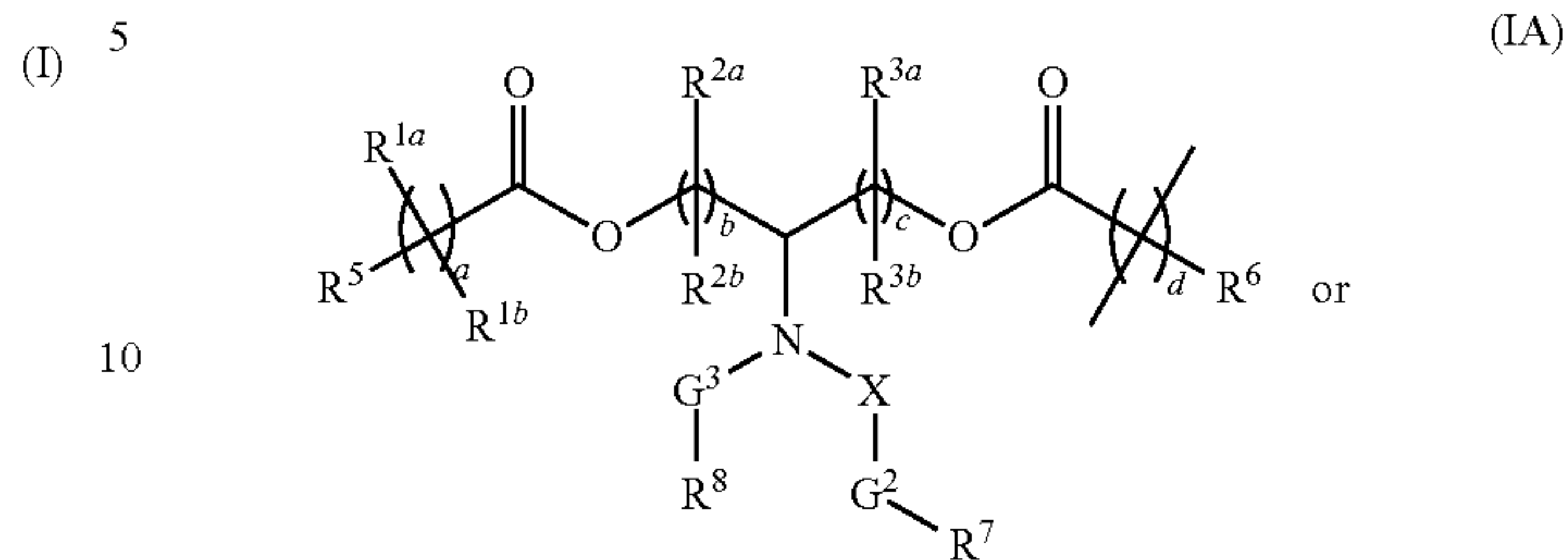
X is $-(C=O)-$ or a direct bond; and

a, b, c and d are each independently an integer from 1 to 24;

wherein each methyl, alkyl and alkylene is independently optionally substituted.

64

2. The compound of claim 1, having one of the following structures (IA) or (IB):



or a pharmaceutically acceptable salt, tautomer or stereoisomer thereof.

3. The compound of claim 1, wherein G^1 is C_2 - C_3 alkylene or C_4 - C_6 alkylene.

4. The compound of claim 1, wherein G^2 is C_2 - C_3 alkylene or C_3 - C_4 alkylene.

5. The compound of claim 1, wherein X is a direct bond.

6. The compound of any one of claim 1, wherein R^7 is $-O(C=O)R^{10}$ or $-(C=O)OR^{10}$.

7. The compound of claim 6, wherein R^{10} is linear C_6 - C_{10} alkyl.

8. The compound of claim 6, wherein R^{10} is methyl.

9. The compound of claim 6, wherein R^{10} is branched C_{10} - C_{15} alkyl.

10. The compound of any one of claim 1, wherein R^7 is $-NR^9(C=O)$ or $-(C=O)NR^9R^{10}$.

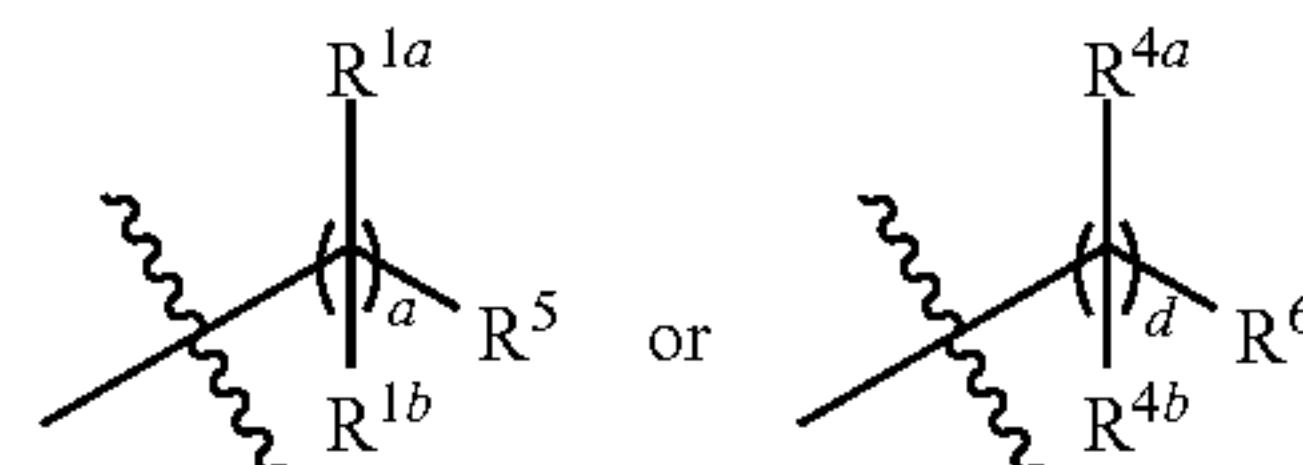
11. The compound of claim 10, wherein R^9 is H.

12. The compound of claim 10, wherein R^9 and R^{10} are each independently C_6 - C_{10} alkyl.

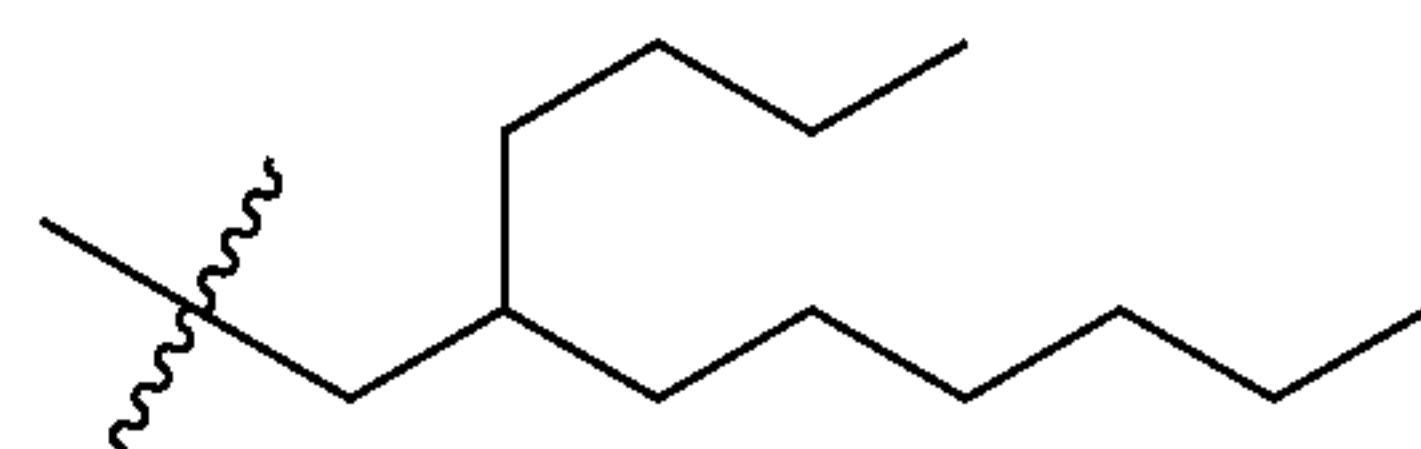
13. The compound of claim 1, wherein R^{1a} , R^{1b} , R^{2a} , R^{2b} , R^{3a} , R^{3b} , R^{4a} and R^{4b} are, at each occurrence, independently H or C_1 - C_{12} alkyl.

14. The compound of claim 13, wherein at least one of R^{1b} and R^{4b} is C_1 - C_8 alkyl.

15. The compound of claim 1, wherein

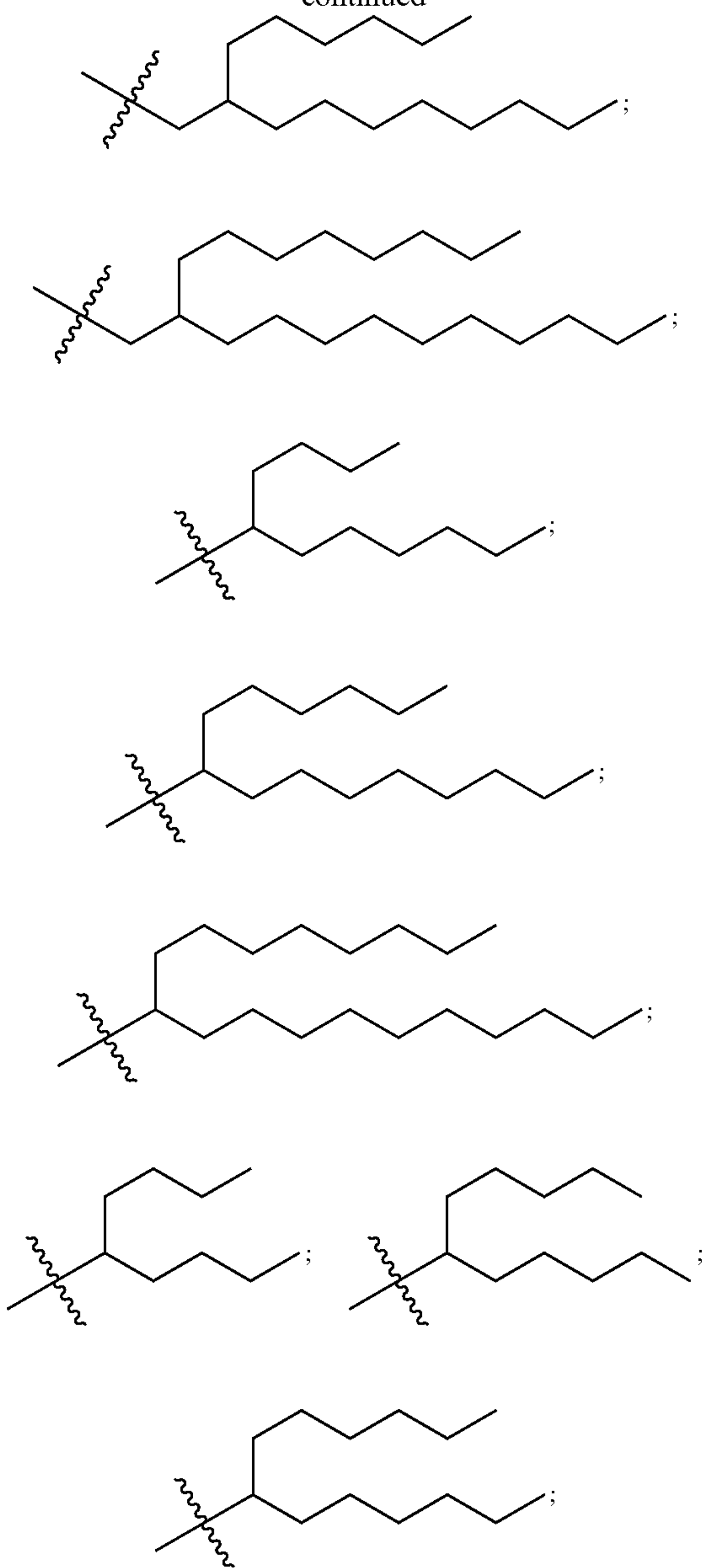


or both, independently has one of the following structures:



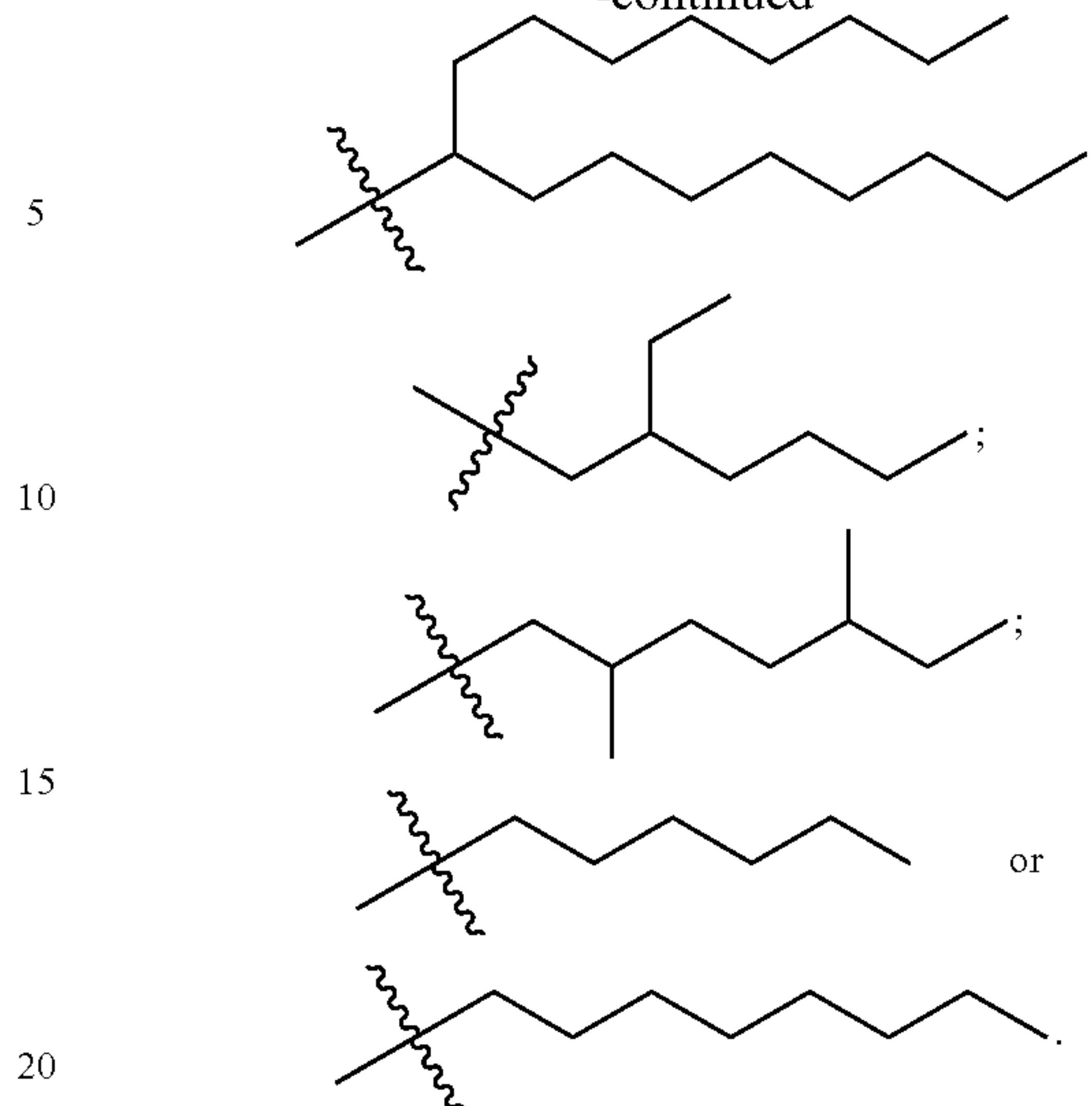
65

-continued



66

-continued



16. The compound of claim 1, wherein a, b, c and d are each independently an integer from 2 to 12.

17. The compound of claim 1, wherein R^8 is $-N(R^{11})(C=O)R^{12}$.

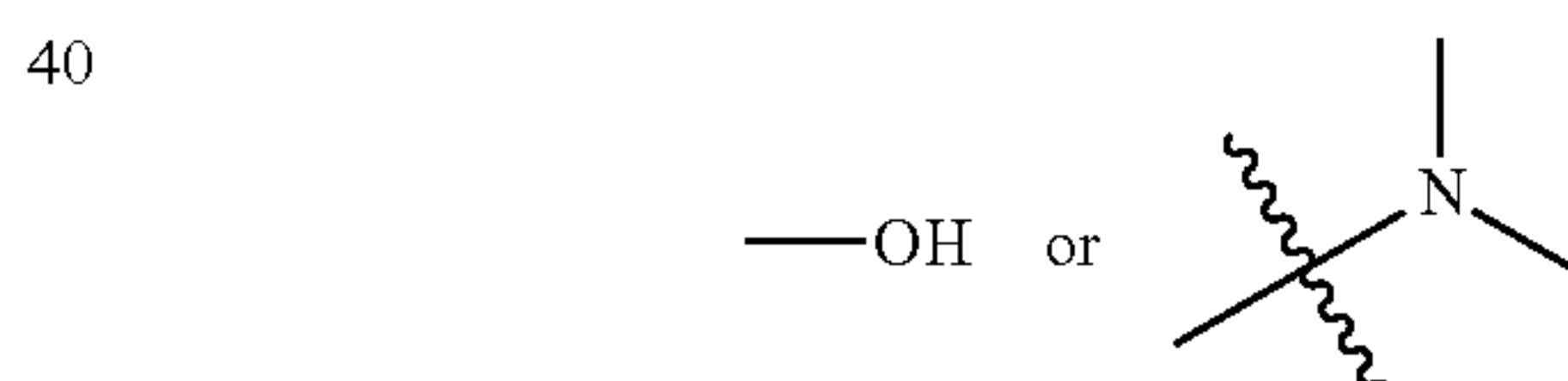
18. The compound of claim 1, wherein R^8 is $-(C=O)N^{11}R^{12}$.

19. The compound of claim 1, wherein R^8 is $-NR^{11}R^{12}$.

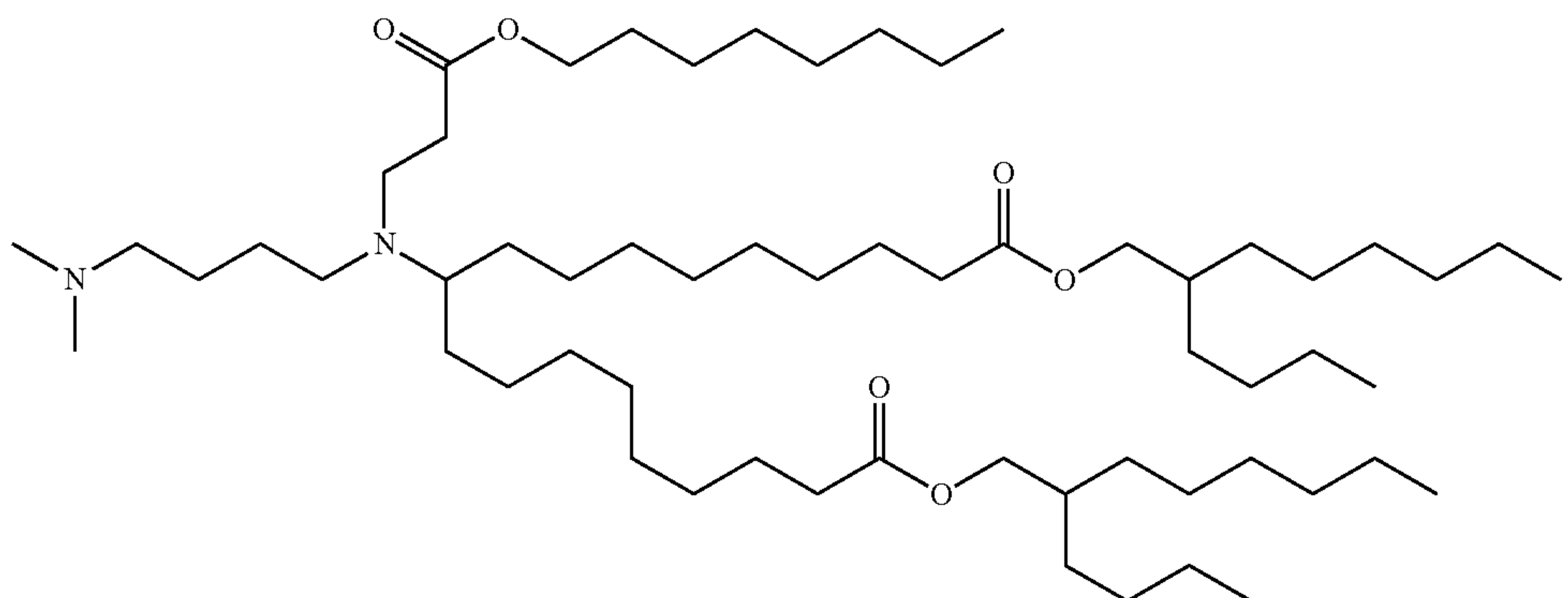
20. The compound of claim 1, wherein R^8 is $-(C=O)OR^{12}$.

21. The compound of claim 20, wherein R^8 is $-O(C=O)R^{12}$.

22. The compound of claim 1, wherein R^8 has one of the following structures:



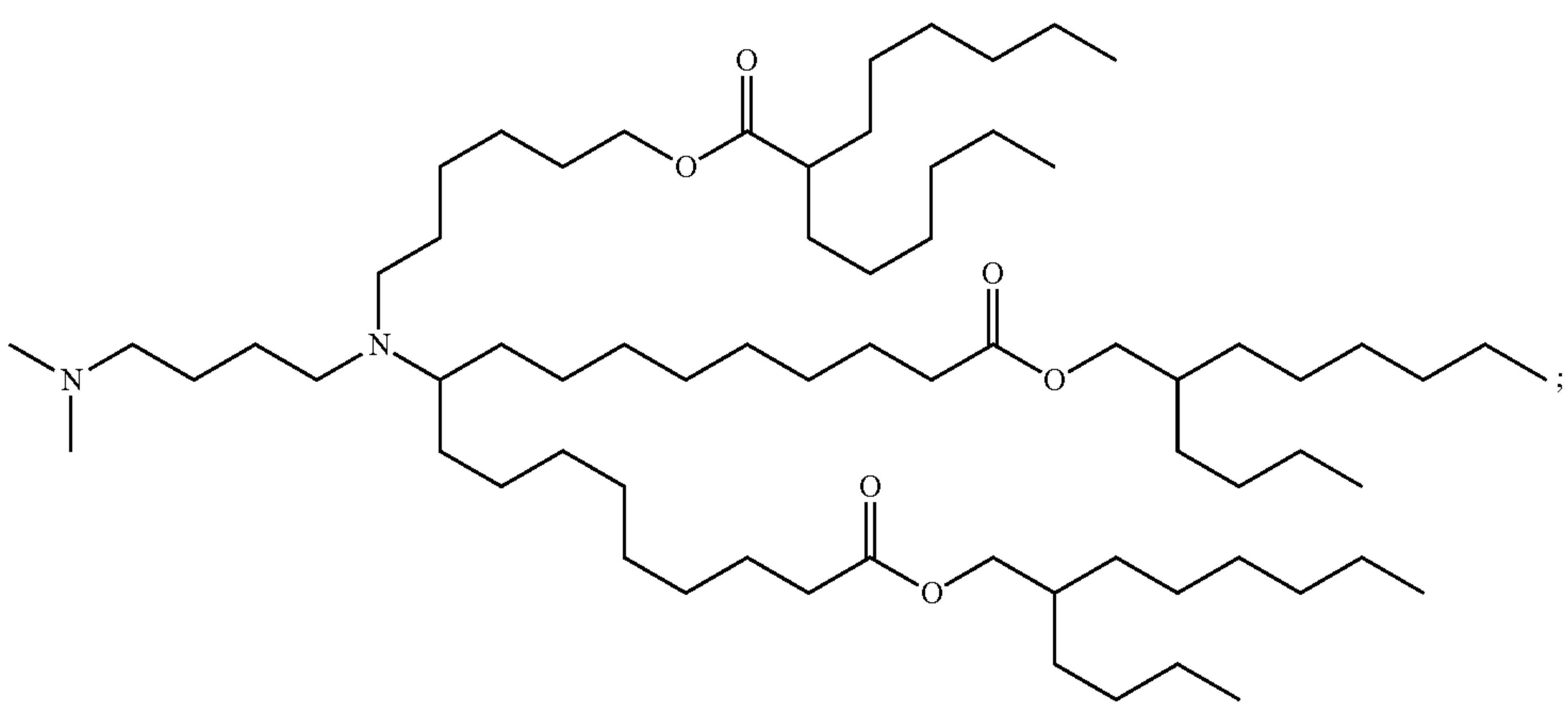
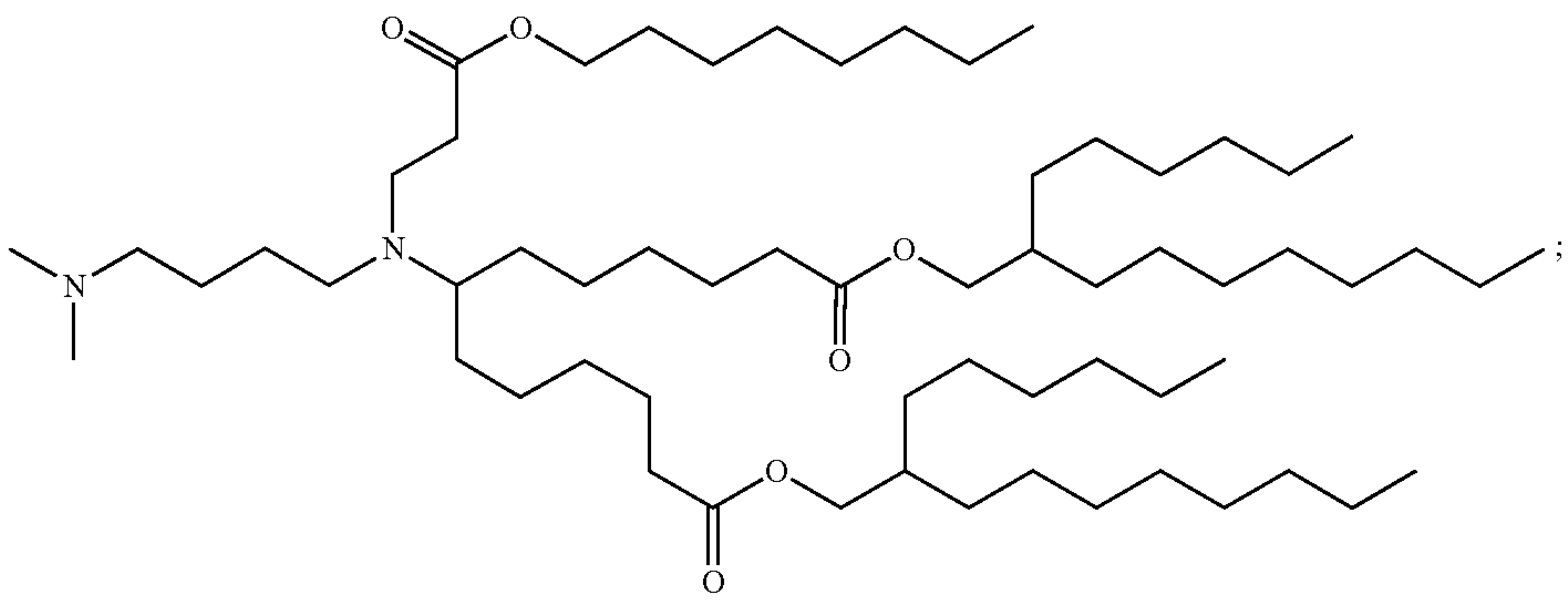
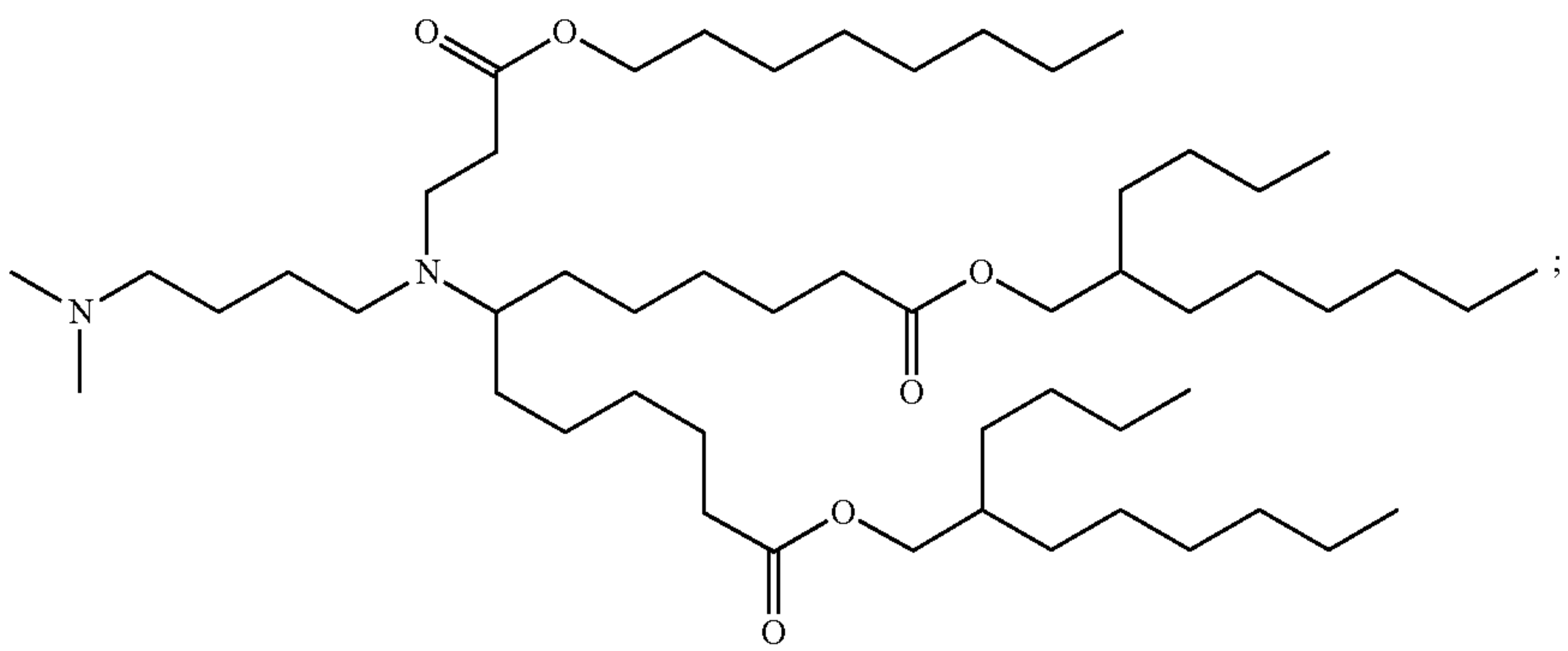
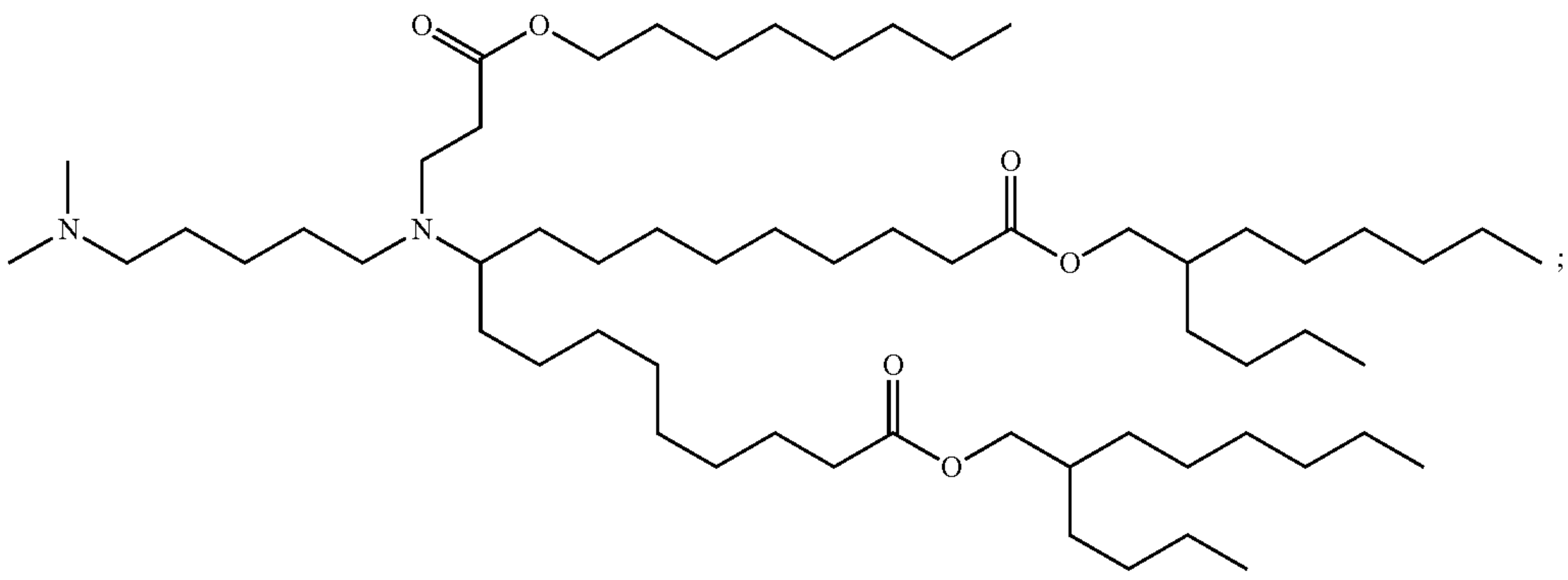
23. The compound of claim 1, having one of the following structures:



67

68

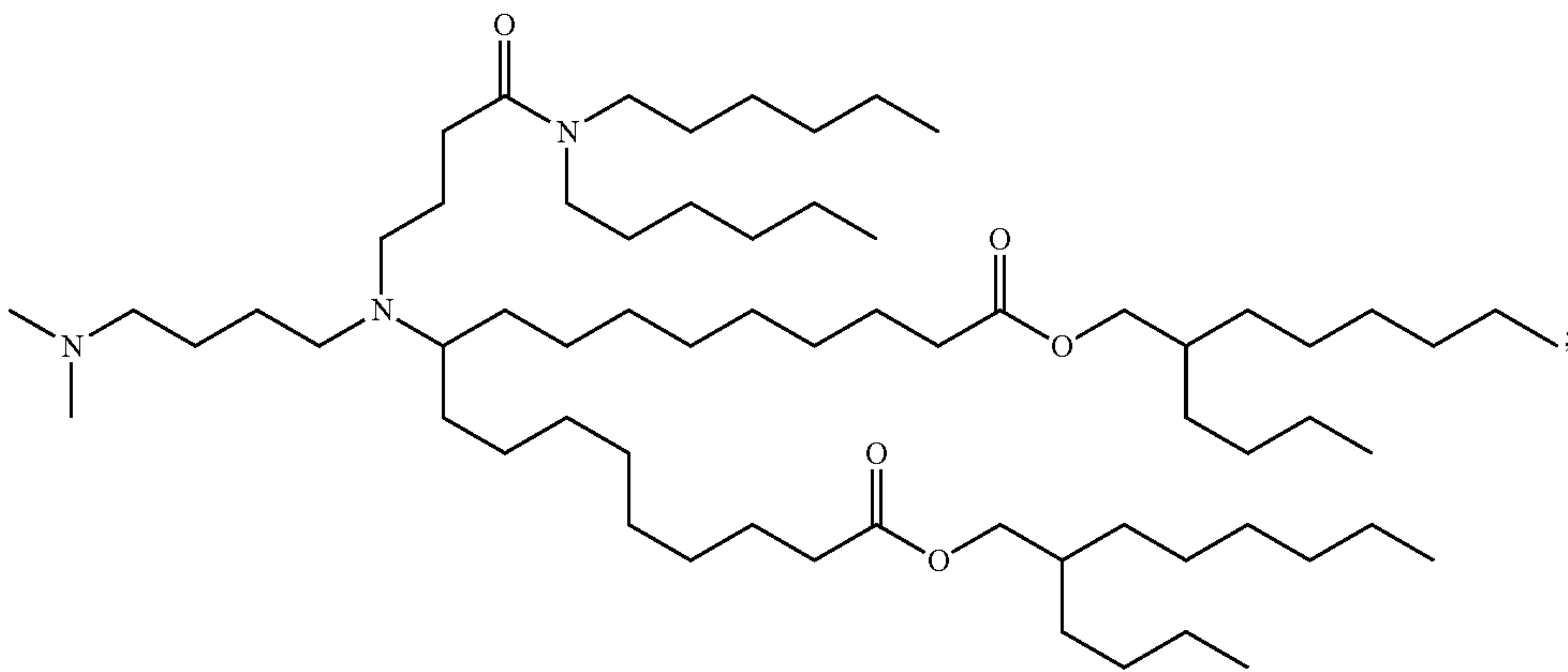
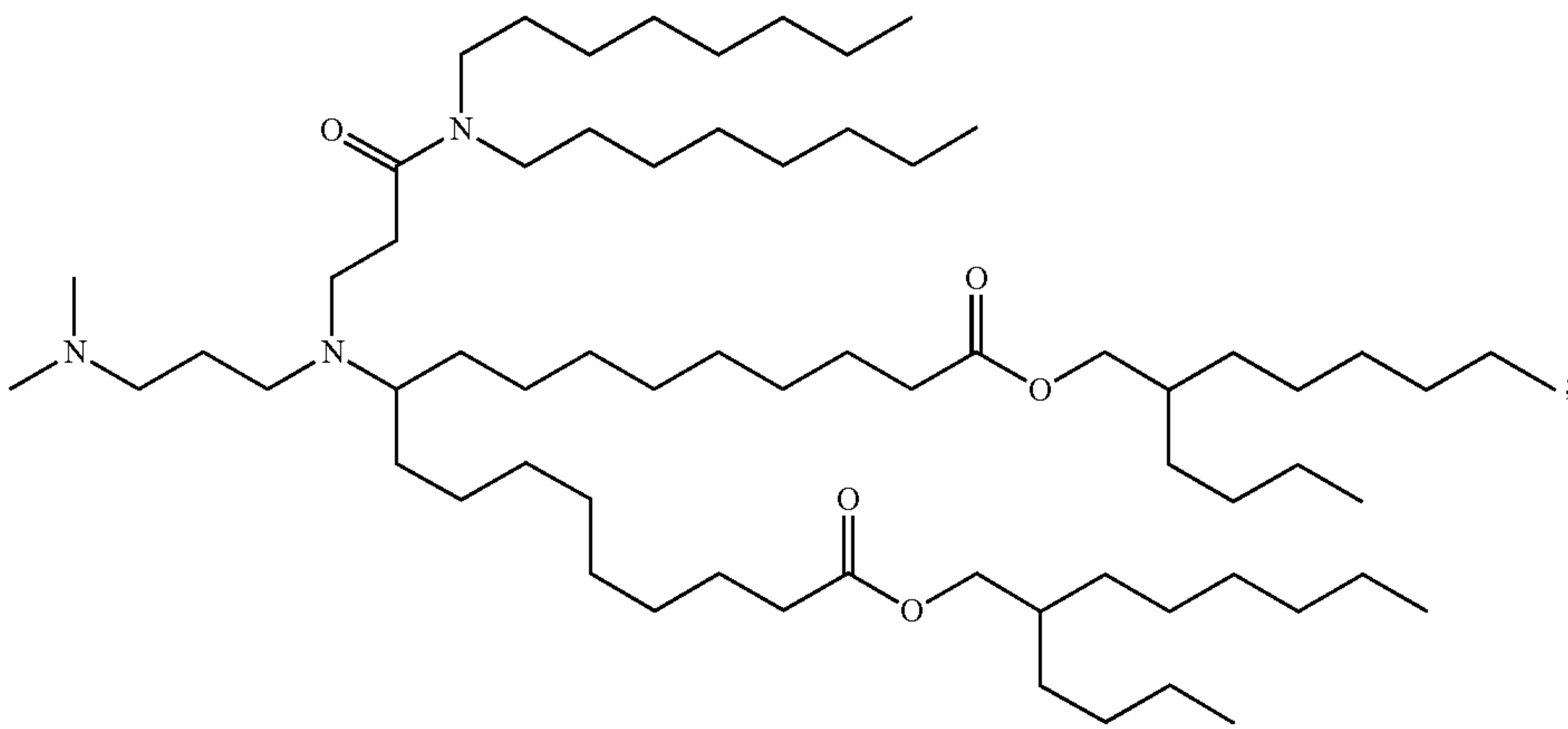
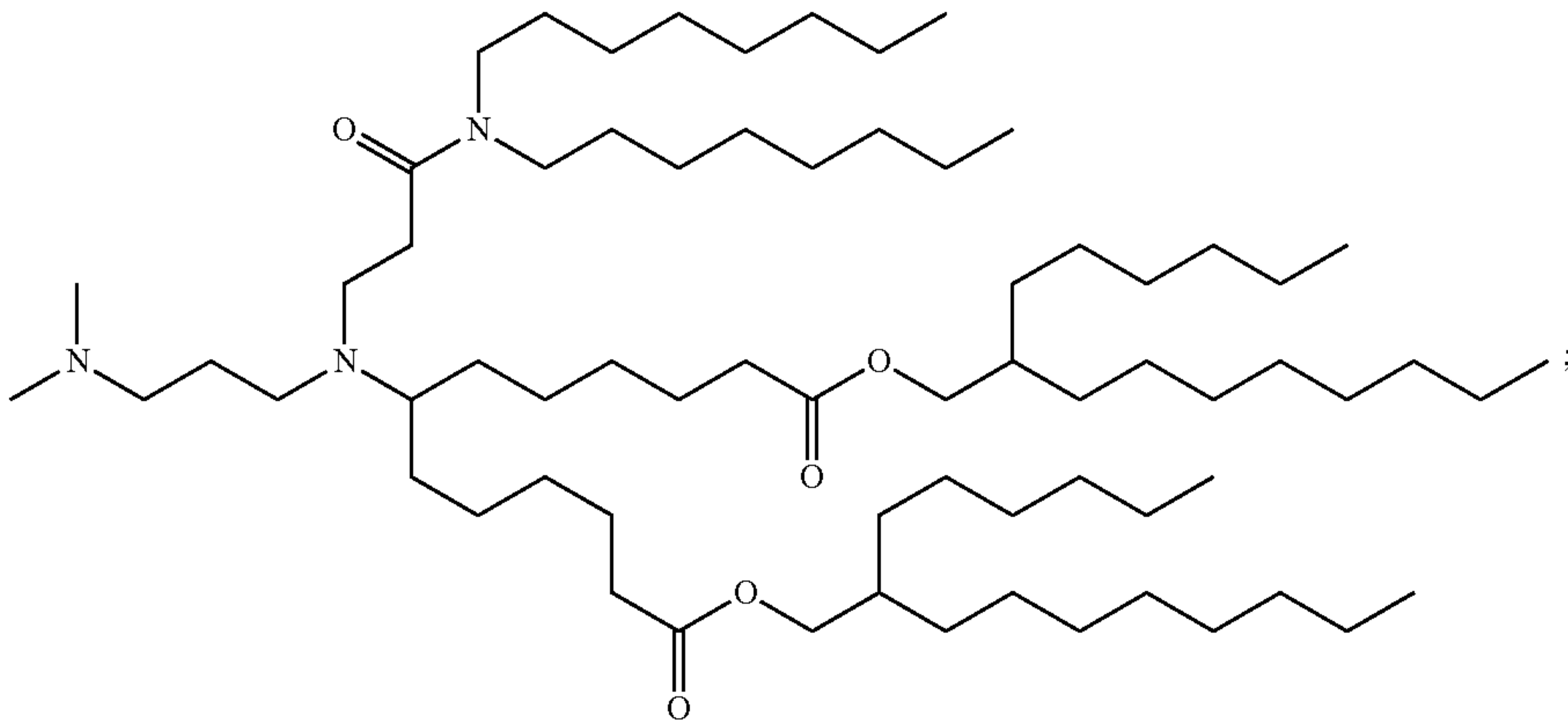
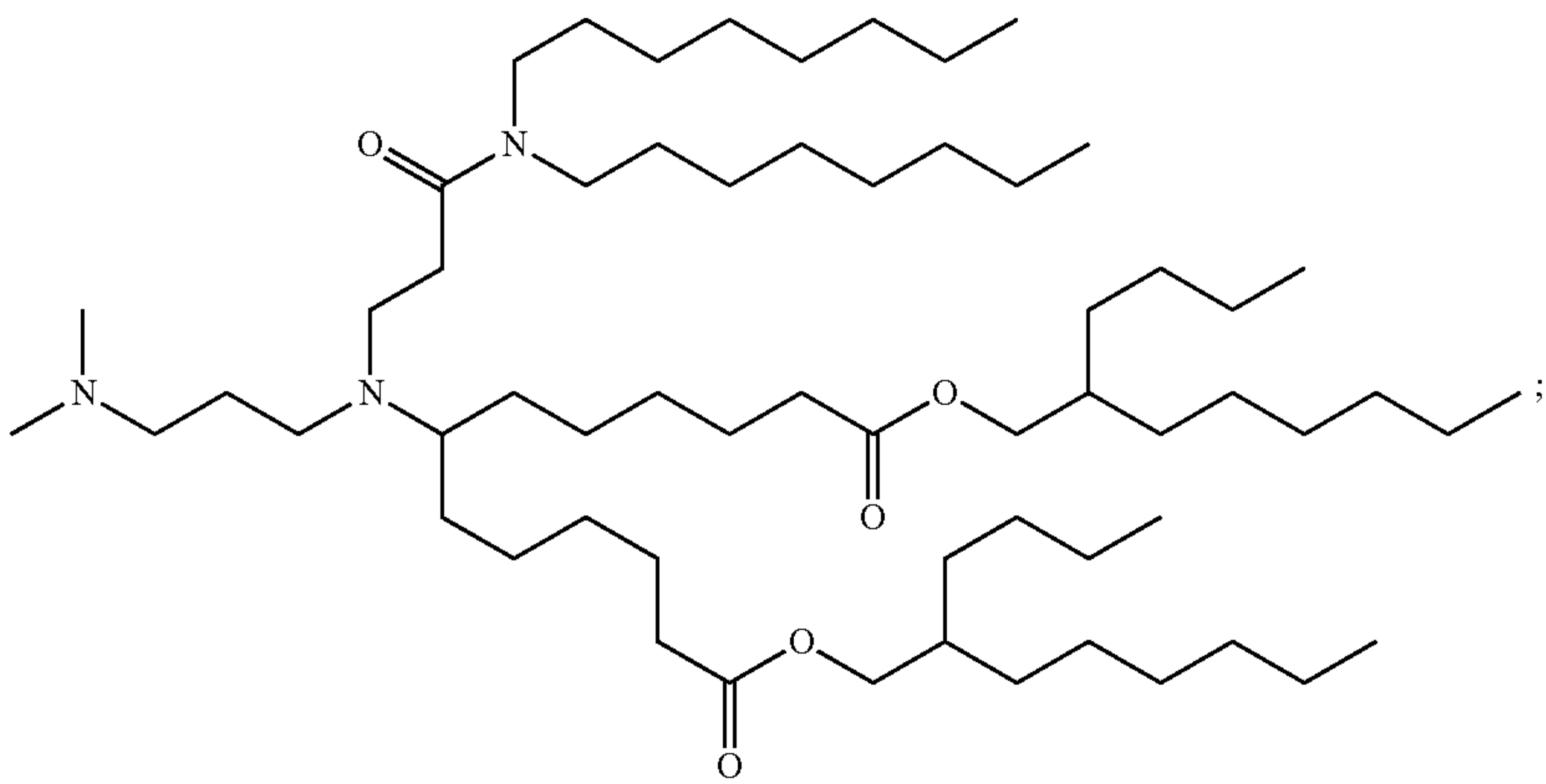
-continued



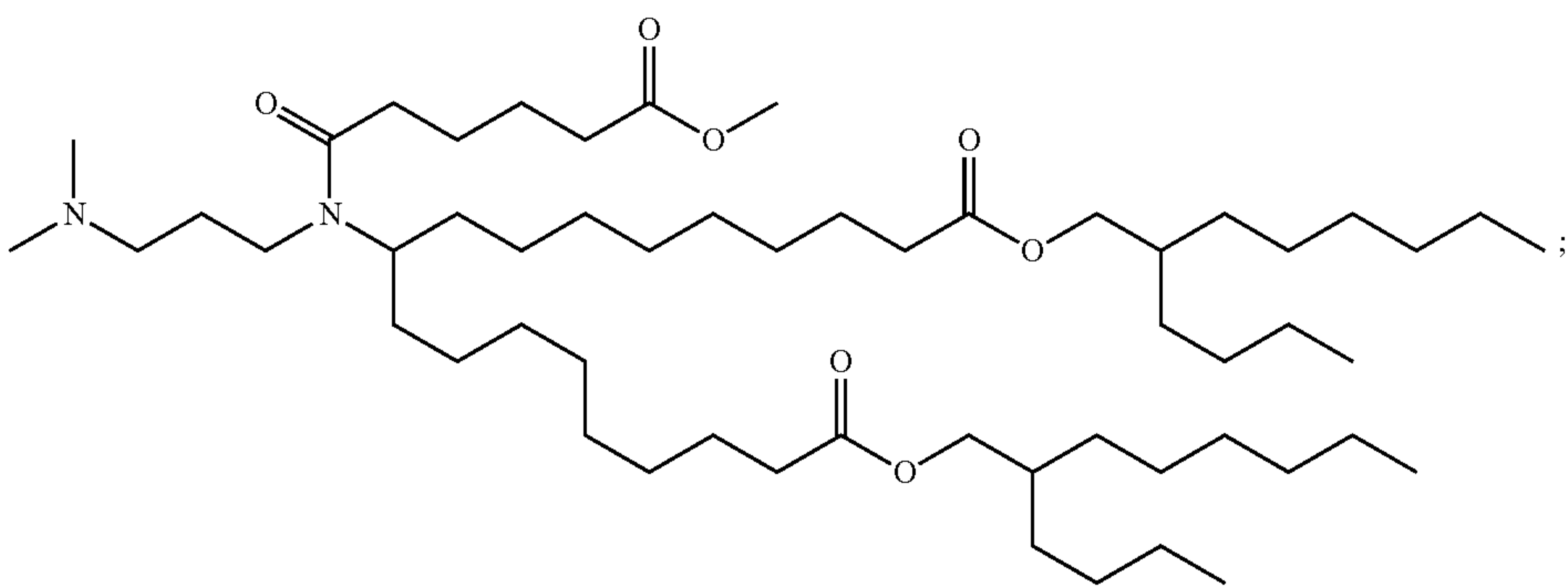
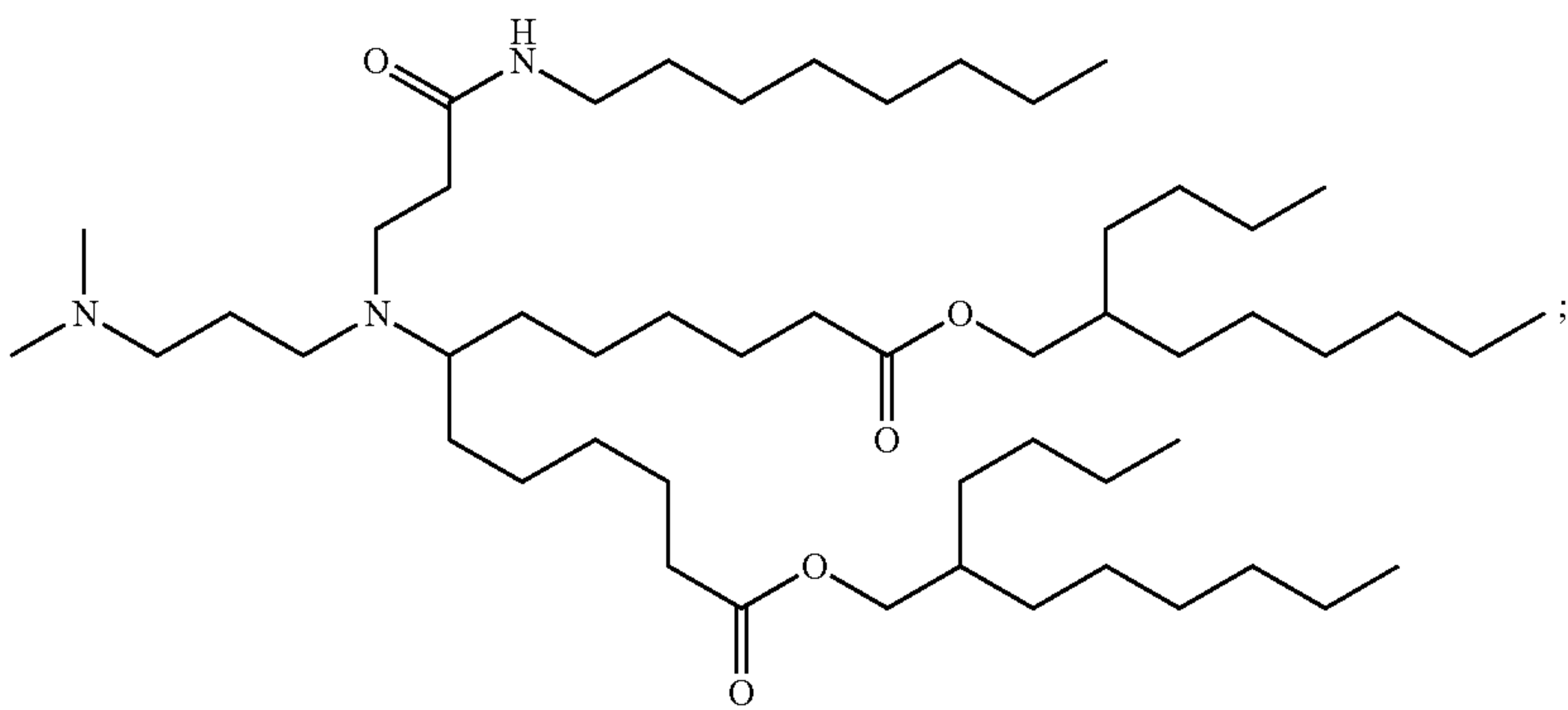
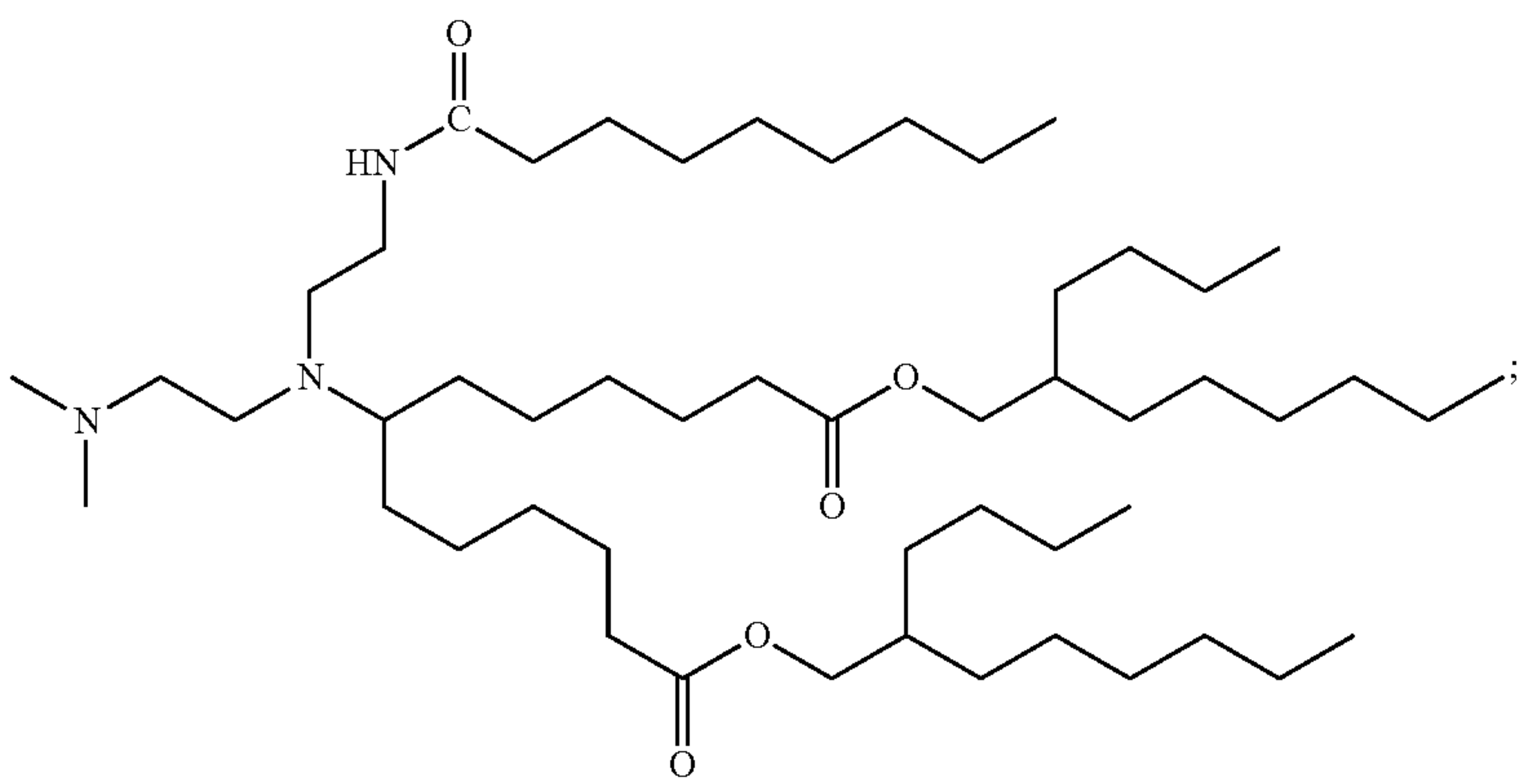
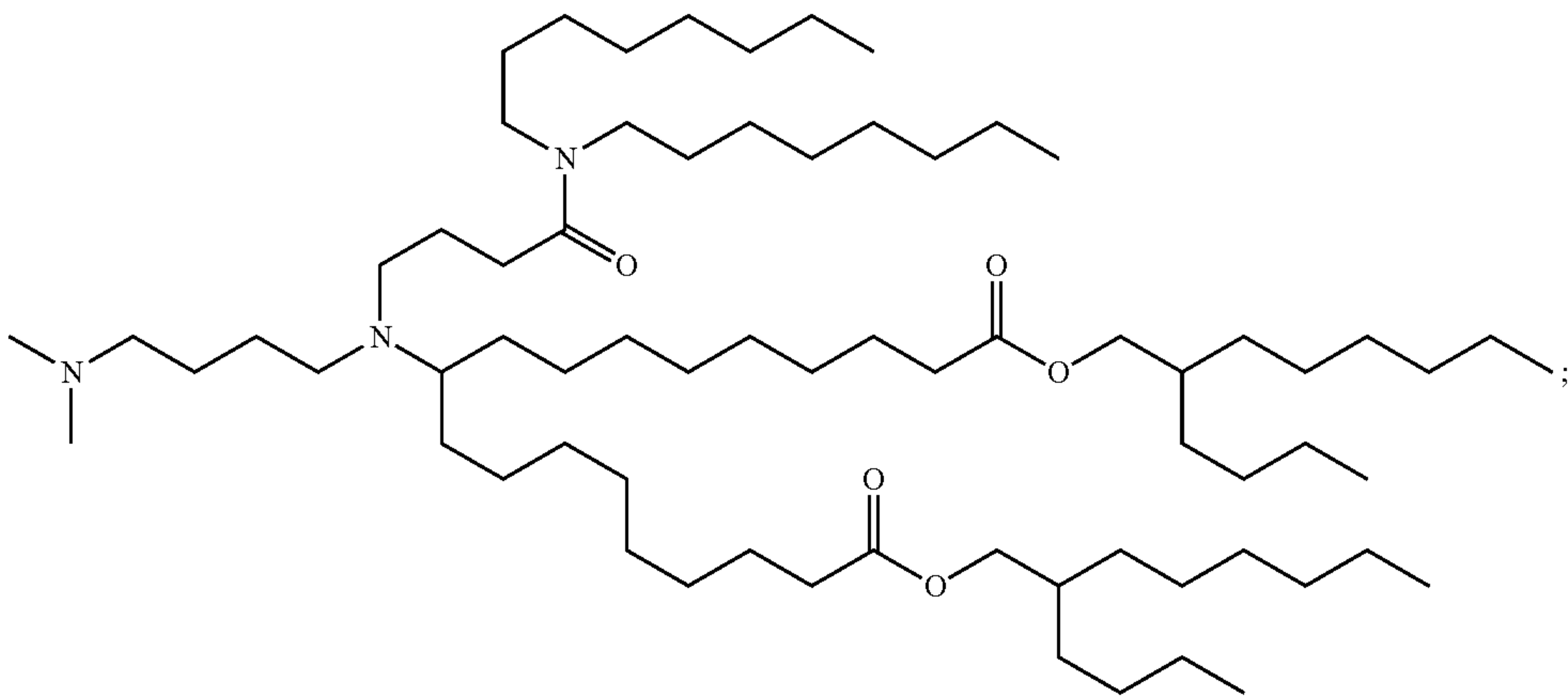
69

70

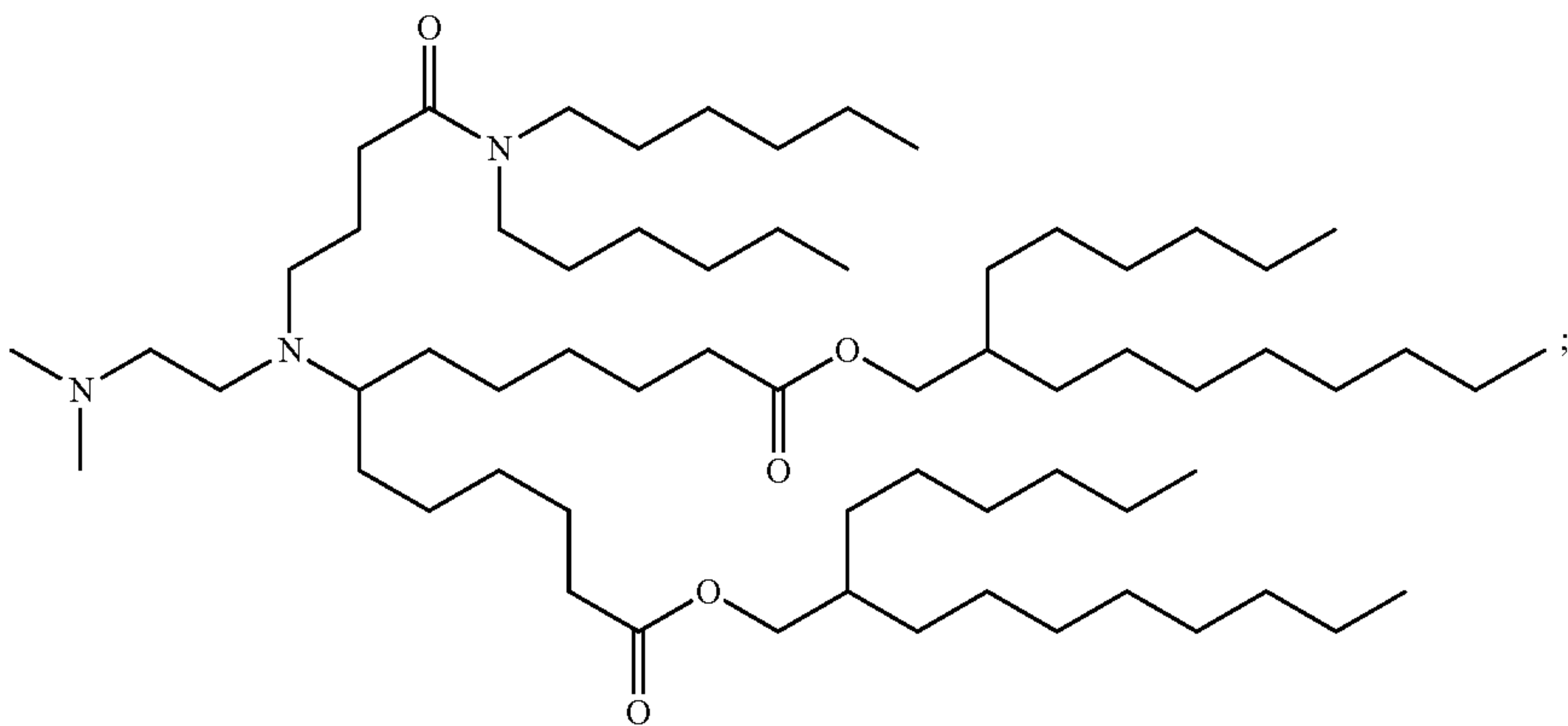
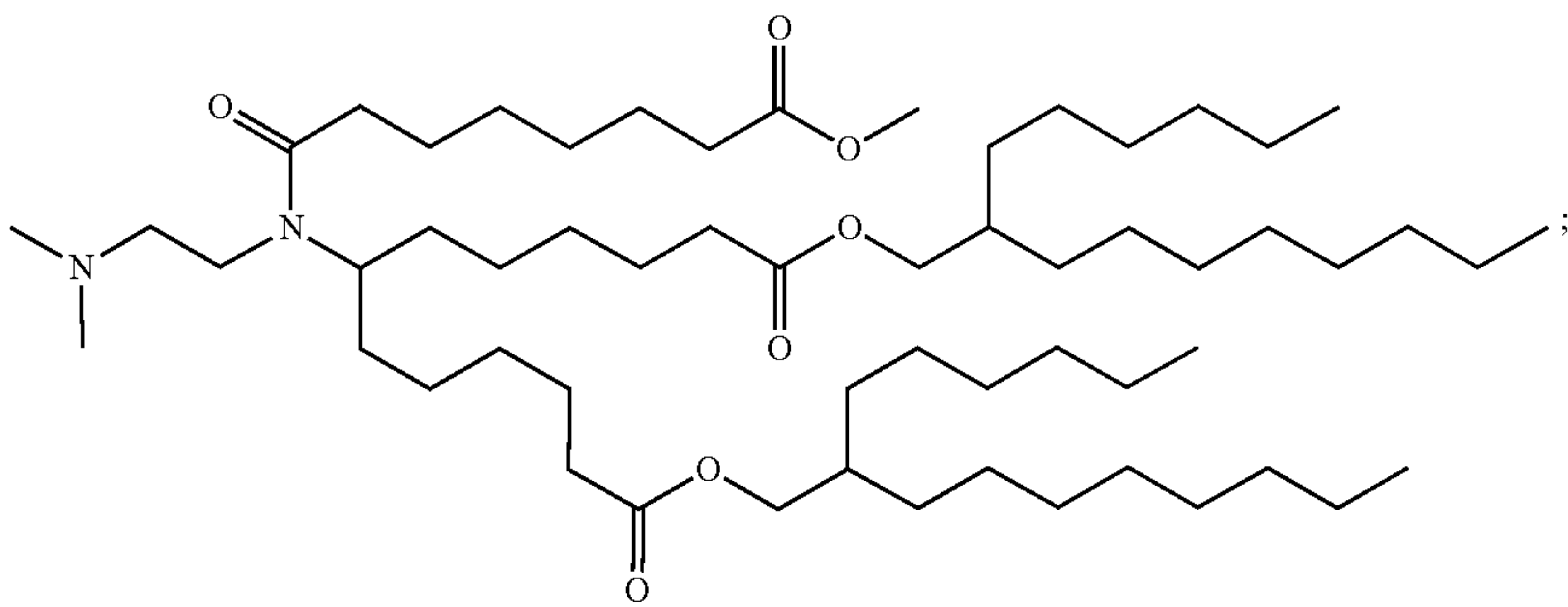
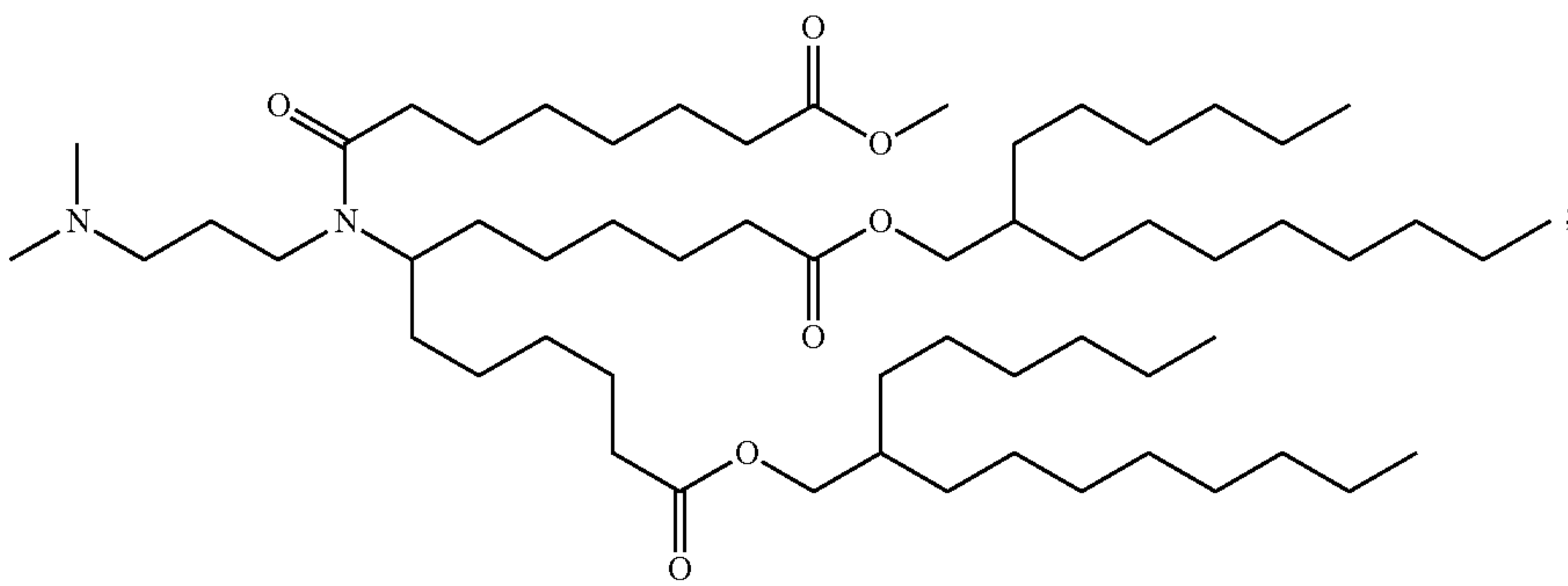
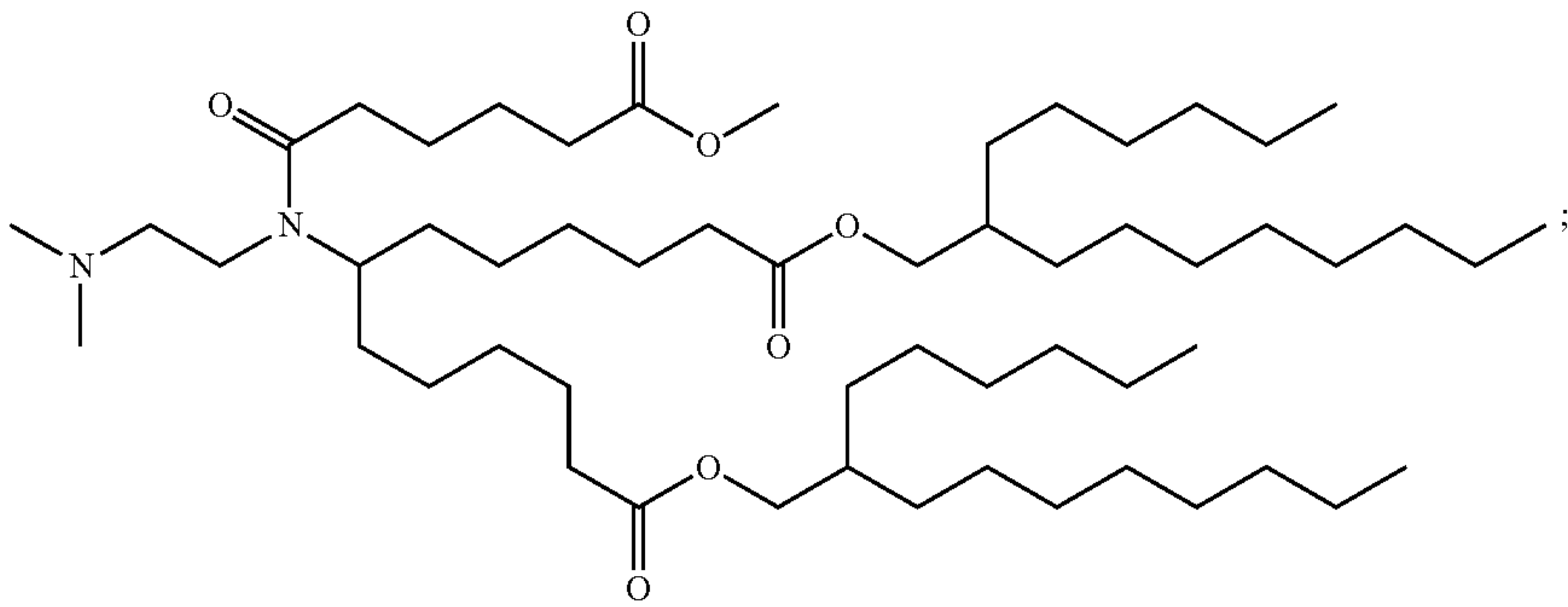
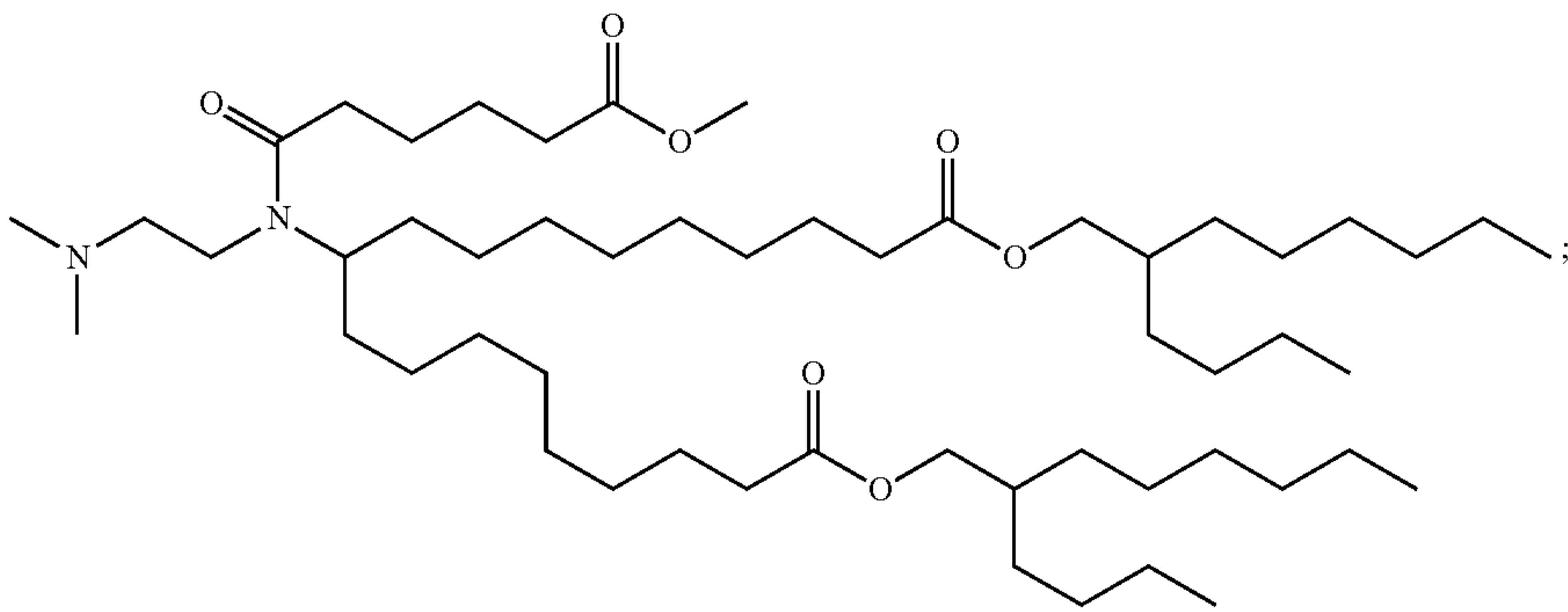
-continued



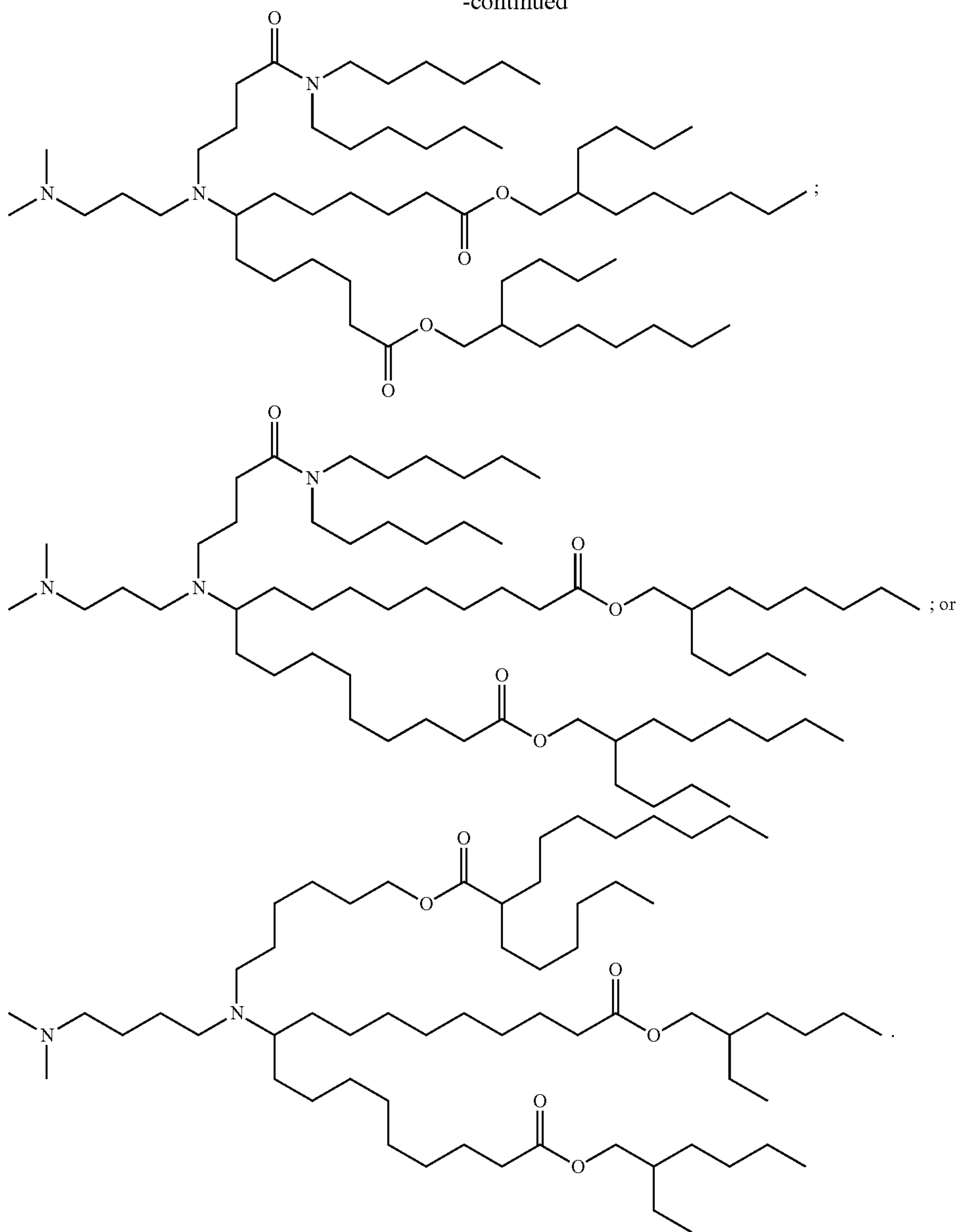
-continued



-continued



-continued



24. A lipid nanoparticle comprising the compound of claim 1 and an mRNA encoding a protein.

25. A pharmaceutical composition comprising the lipid nanoparticle of claim 24 and a pharmaceutically acceptable diluent or excipient.

26. A method for expressing a protein in a patient in need thereof, the method comprising administering the lipid nan-

50
 nanoparticle of claim 24 to the patient, wherein the protein encoded by the mRNA is expressed in the patient.

27. A method for vaccinating a patient in need thereof against a viral pathogen, the method comprising administering the lipid nanoparticle of claim 24 to the patient, wherein the protein is a viral antigen.

* * * * *